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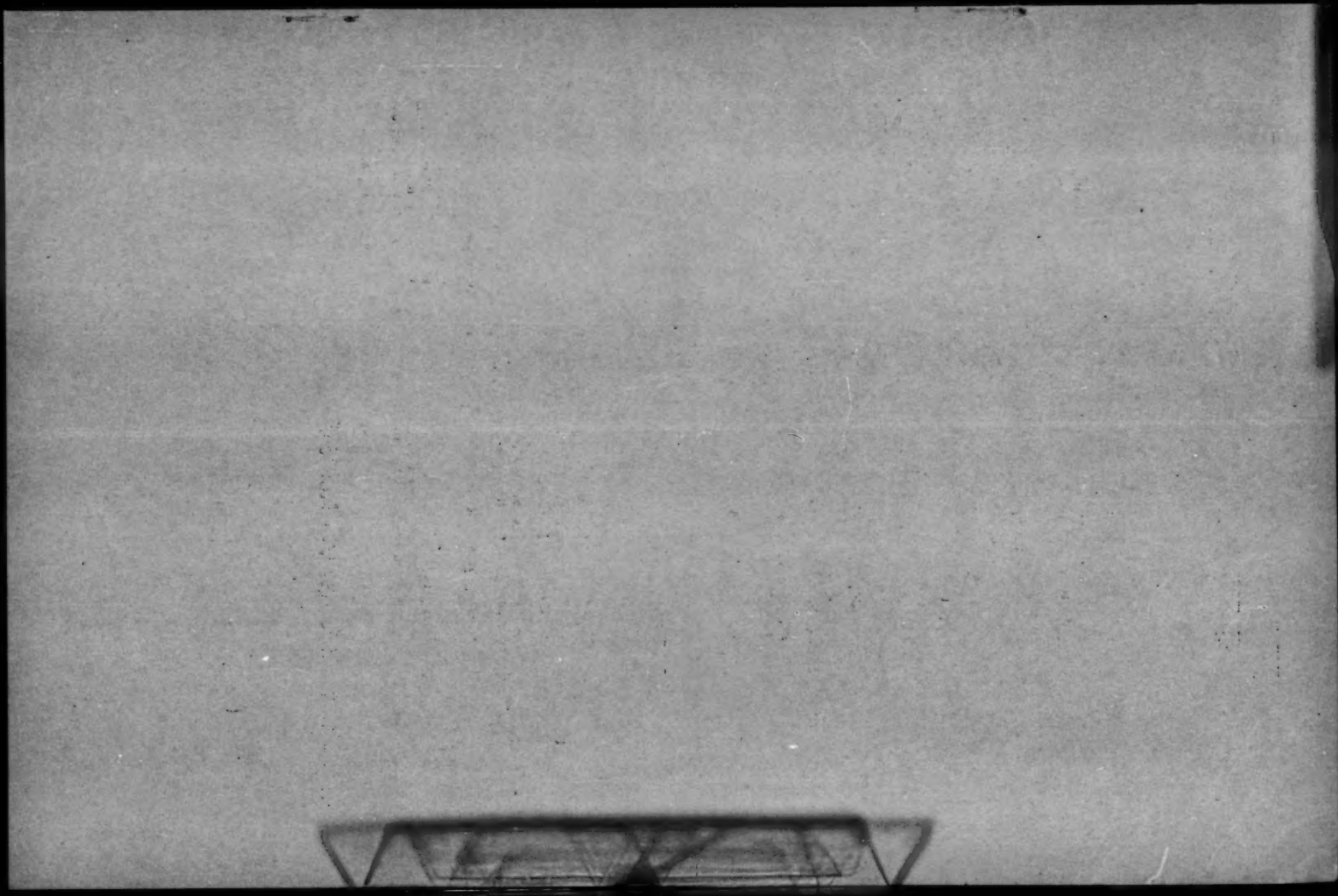

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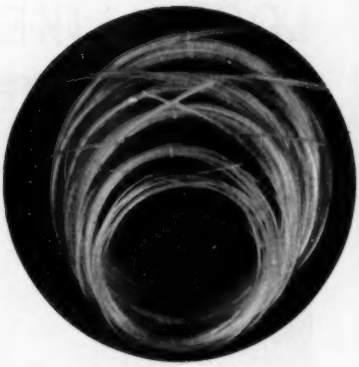

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# Attempts To Produce Bovine Hyperkeratosis

CARL OLSON, Jr., D.V.M., M.S., Ph.D., and R. H. COOK, D.V.M., M.S.

*Lincoln, Nebraska*

FOR A QUICK understanding of this paper, the authors suggest first reading the sections on Literature, Discussion, and Summary. If further interested in details of the experiments, proceed with sections on Methods and Results.

## LITERATURE

Bovine hyperkeratosis (X disease, hyperkeratosis, proliferative stomatitis, and esophagitis) has been recognized as a disease entity since the report of Olafson[3] in 1947. The etiology of the disease has remained obscure, despite numerous attempts at transmission or production of bovine hyperkeratosis.

A brief résumé of the reports on attempts to produce the disease is in order. Olafson[7] was unable to cause the disease by large transfusions of whole blood from affected to test animals, the introduction of new animals into a herd, intimate contact between diseased and test animals in small crowded stalls, and scarification of oral mucosa of test animals followed by contact with sick animals. Neither was there evidence of transfer of the disease to young calves suckling the same cow with a calf that had lesions in the mouth. Inoculation onto the scarified oral mucosa with mouth lesion material from cases of bovine hyperkeratosis caused the development of wartlike lesions in the mouths of test calves (Olafson and Fincher, cited by Hagan[5]). The Annual Report of the New York Veterinary College for 1944-45[6] indicated that material taken from ulcers of the mouths of cattle with bovine hyperkeratosis and placed on scarified buccal mucosa of normal animals led to the development of soft whitish papilloma-like lesions within a week or so. Gibbons[2] reported negative results from attempted transmission by means of association, feeding of material from lesions (the type of lesion or material was not specified), and the inoculation with blood. He did state that rubbing exudate from lesions of the mouth onto the scarified tongue of a calf would, at times, produce a local reaction somewhat similar to the original lesions, but no general symptoms of bovine hyperkeratosis developed.

In 1948, Gibbons[3] reported an experiment in which 2 calves, 6 and 3 months old, were exposed to a calf acutely sick with bovine hyperkeratosis. In seven and eight days, the healthy calves developed diarrhea and a slight temperature. Ulcers and pustules developed in the mouth and on the muzzle in eight days. The diarrhea lasted from two to three weeks. No skin lesions developed and the calves recovered. From 1 of these calves, blood was obtained and injected subcutaneously into a 2-month-old calf. This calf developed superficial erosions in the mouth and a diarrhea from which it later recovered. Another calf, 9 months old, was given blood subcutaneously. In five days, it had a reddened mouth and, eight days later, an elevation of temperature with diarrhea and erosions of the mucosa in the mouth. No lesions of the skin developed and the calf died two months later from parasitism (coccidiosis and hookworms).

In 1949, Gibbons[4] (apparently reviewing these latter experiments) briefly described the reaction as follows. The subcutaneous injection with blood from a naturally infected calf caused, seven to eight days later, a temperature of 104.0 to 105.6 F., red congested and superficial erosions of the mucosa in the mouth, diarrhea, and leukopenia. He stated that this condition did not spread by contact and the calves recovered spontaneously in three to five days. Fox, cited by Gibbons,[4] is reported to have produced a stomatitis and esophagitis with infective material from a case of X disease. The condition was not associated with lacrimation, skin lesions, or proliferative le-

From the Department of Animal Pathology and Hygiene, University of Nebraska, Lincoln.

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sions as seen in the natural disease. Beck[1] records a negative result in 2 cows inoculated with material from 4 affected heifers. He does not state the type of material used for inoculation.

It therefore appears from the literature that two conditions have been produced in experimental calves with material coming from cases of bovine hyperkeratosis. One of these conditions was a transient papilloma-like lesion developing on the oral mucosa following inoculation with material from a similar lesion of a spontaneous disease on the scarified oral mucosa. The second condition was a transient elevation of temperature with diarrhea, having an incubation period of a little more than a week and a course of only a few days. Lesions of the mouth associated with this second condition seemed to be superficial erosions, hyperemia, stomatitis, and esophagitis. The etiological factor of this second condition appeared to be contained in the blood.

In none of the attempts at experimental production of bovine hyperkeratosis was there development of skin lesions, lacrimation, or a train of long-continued symptoms. The observations by Olson, Cook, and Brouse[5] on an outbreak of hyperkeratosis at Valentine, Neb., suggested that some unknown factor or factors in the feedstuffs increased the severity of symptoms and lesions of the disease. During the study of this outbreak, certain experiments were initiated in an attempt to produce the disease in healthy calves. The purpose of this report is to relate these experiments and the results obtained.

## METHODS

The conditions and circumstances surrounding the outbreak of bovine hyperkeratosis have been described in detail.[5] A supply of pelleted supplemental feed that had been fed to the calves that developed the disease was available. One type of pelleted feed consisted of dehydrated alfalfa. This had been fed to a group of 10 calves in lot 6 from November, 1948, to February, 1949. All 10 calves were affected with the disease by February and 8 died during February, March, and April. This supplemental feed will be referred to as lot 6 pellet. A second, supplemental pelleted feed was composed of 97.5 per cent dehydrated alfalfa and 2.5 per cent dicalcium phosphate. This supplemental feed was given to two lots of calves (2 and 14) from November, 1948, to March, 1949. All 10 calves in lot 2 developed the disease and died during March, April, May, and July of 1949. Ten calves in lot 14 received the same feedstuff, and all developed bovine hyperkeratosis from which 8 died during April, May, June, and October, 1949. This pelleted supplement will be referred to as lot 2 pellet. A third pelleted supplement consisted of 96 per cent dehydrated alfalfa and 4 per cent dicalcium phosphate. This was fed from November to March to 10 calves in lot 5, all of which developed bovine hyperkeratosis. Five calves died in February, April, May, and August, 1949. This supplement will be called lot 5 pellet.

The general pattern of the above results was evident by June, 1949. It seemed advisable to initiate some tests while the outbreak of the disease was still going on. It was possible to obtain calves similar to the original calves. These were from the same herd and part of the same crop of calves as were involved in the outbreak. The difference between the original group of calves which suffered the outbreak of bovine hyperkeratosis and the calves obtained in June, 1949, as test calves was that the latter had been wintered by a rancher and remained healthy. On June 15, 1949, 35 new calves were delivered to the Experiment Substation at Valentine, Neb. These calves were examined individually on June 17 and then placed in five lots. The lots were those previously used for calves which had bovine hyperkeratosis. No attempt was made to clean or disinfect these lots, except to remove some of the accumulated manure. The new calves were placed in the lots immediately after sick calves had been removed. Four of the lots (11, 12, 13, and 14) were adjacent to one another. Lots 11 and 12 and lots 13 and 14 had water tanks in common. Lot 15 that lay between lots 14 and 16 was left vacant. Lot 16 was occupied by new test calves and was somewhat isolated by lot 15. Five new calves were placed in each of lots 11, 12, and 13. Ten new calves were placed in each of lots 14 and 16. Tests for contact transmission of the disease were conducted in lots 11, 12, 13, and 14 by adding calves selected from the group which was undergoing the outbreak of bovine hyperkeratosis. Five calves in which there had been red crescentic lesions of the oral mucosa predominating and still present were added to lot 11. Five calves with papilloma of the mouth as the predominating lesion and little or no hyperkeratosis of the skin were added to lot 12. Five calves in which hyperkeratosis of the skin predominated and papillomas of the mouth were either absent or mild were added to lot 13. Five calves selected from the original group in which bovine hyperkeratosis was marked both from the standpoint of lesions of the skin and papillomas of the mouth were added to lot 14. Some of the sick calves placed in contact with the new calves died or recovered, and other calves with a comparable status of the disease were added to test lots 11, 12, 13, and 14. These conditions were maintained for thirty weeks. The test calves were kept in their allotted pens for ten weeks after the contact calves were removed. No calves from the original group with bovine hyperkeratosis were added to lot 16. The ration for calves in lots 11, 12, and 13

consisted of soybean meal (1 lb. per calf daily) as a supplement to prairie hay. Lots 14 and 16 received prairie hay and a supplement of lot 2, 5, and 6 pellets. These were fed at the rate of 1 lb. per calf daily, and each type of pellet was fed on alternate days. The pellets were fed to the calves of lots 14 and 16 for seventy-four days. The calves were then fed soybean meal as a supplement to the prairie hay.

In order to have some control on environment as a possible factor, 10 new calves obtained at the same time and from the same source as the other test calves were put in a pen at Lincoln, Neb. These calves had no contact with sick calves. They were fed lot 2 pellets for seventy-four days and then continued on soybean meal as a supplement to prairie hay.

Beginning January 20, 1949, 2 calves (51 and 139) at Valentine were selected for a second course of feeding with lot 2, 5, and 6 pellets. These replaced the soybean meal supplement until April 24 (94 days), when soybean supplement was again resumed. Three calves (48, 144, and 236) at Lincoln were selected for an additional course of feeding with lot 2 pellets, as a replacement for soybean meal supplement. This course of feeding was begun March 27, 1950, and continued for seventy-four days. Four of the 10 calves in Lincoln were dismissed from the test on March 27, leaving 3 calves as controls for the extra feeding period.

Ten to fifteen physical examinations were made of the individual calves at Valentine and Lincoln during the observation period.

The system for rating and evaluating the different symptoms and lesions of bovine hyperkeratosis developed in a previous study[6] was used in evaluating the results obtained in these trials. By this system, hyperkeratosis of the skin characteristic of bovine hyperkeratosis (fig. 4 and 5) was scored as 10, definite papilloma of the mouth (fig. 1) as 6, lacrimation (fig. 5) as 5, salivation as 5, emaciation as 2, diarrhea as 2, ulceration of the oral mucosa (fig. 1) as 2, a lesion called beginning papilloma (fig. 2 and 3) as 1, elevation of body temperature above 103 F. as 1, and infection about the head or neck, such as abscess of the jaw, as 1. The score for mild lesions or symptoms was reduced by one half. The data from each examination on an individual animal were classified according to this numbering system. The sum of the rating values was considered as the bovine hyperkeratosis score for that particular animal for that specific examination. The score for a given animal over the entire period of observations was obtained by taking the average of the scores for each examination. The average value for the group of animals was determined by averaging the individual scores of the animals.

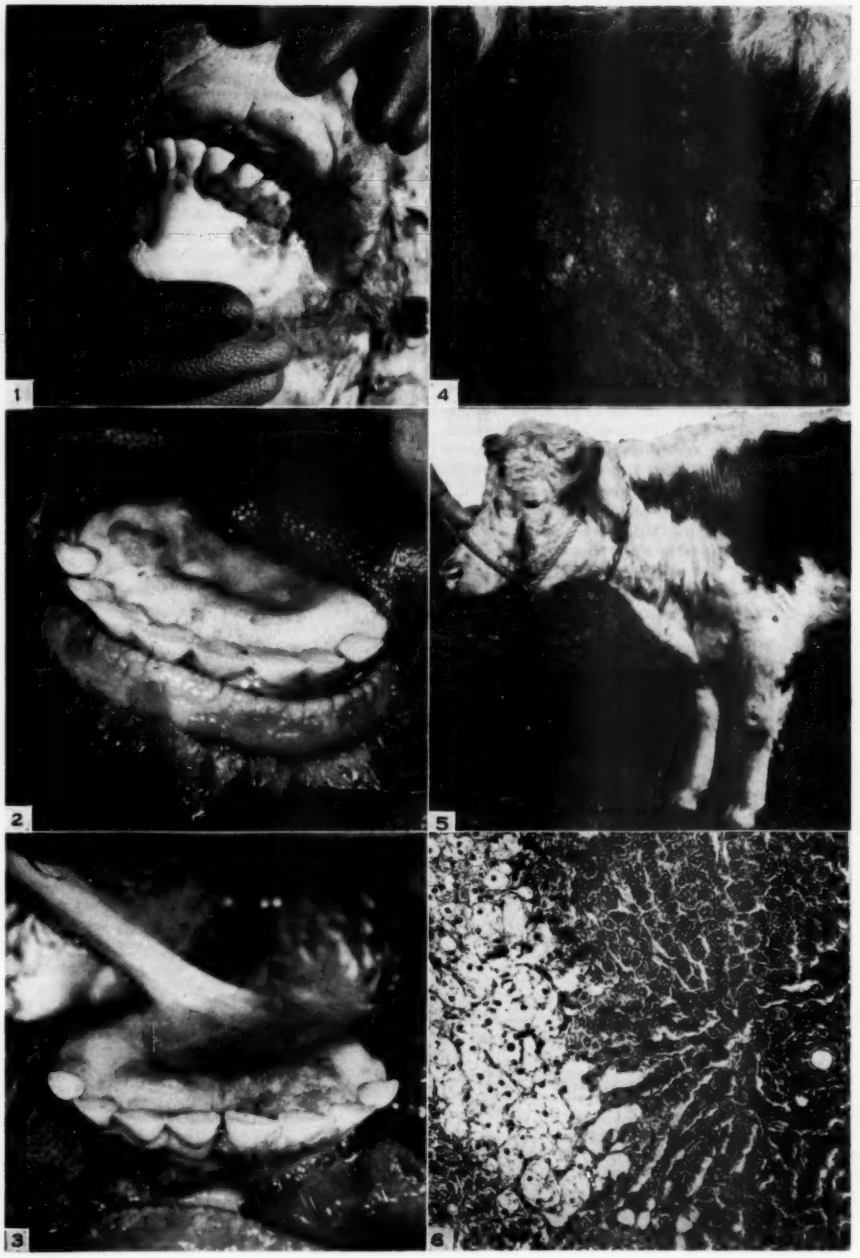
## RESULTS

*Results from Initial Feeding, Contact, and Environmental Exposure.*—Previous studies

TABLE 1—Bovine Hyperkeratosis Scores for Calves During a Forty-Week Attempt to Produce the Disease. Variables Include Contact with Affected Calves (Degree of Illness Varied) and a Suspected Feed Supplement

| Lot | Type calves | No. calves | Ave. bovine hyperkeratosis score | |
|---|---|---|---|---|
| | | | Group ave. | Range (individual ave.) |
| 11 | Contact (red cresentic lesion of mouth) | 6* | 2.7** | 1.4 to 3.9 |
| | Test | 5 | 2.4 | 0.4 to 5.4 |
| 12 | Contact (papilloma of mouth) | 8* | 5.1 | 0.6 to 10.6 |
| | Test | 5 | 1.8 | 0.5 to 2.2 |
| 13 | Contact (skin lesion) | 8* | 7.0 | 2.5 to 14.1 |
| | Test | 5 | 1.8 | 0.5 to 2.9 |
| 14 | Contact (skin and oral lesions) | 9* | 19.7 | 13.2 to 24.3 |
| | Test (suspected feed) | 10 | 4.0 | 1.7 to 6.3 |
| 16 | Test (suspected feed) | 10 | 3.0 | 0.6 to 5.0 |
| Lincoln | Test*** (suspected feed) | 10 | 2.9 | 0.0 to 7.3 |

*No more than 5 in pen at any one time. Changes were required because of recovery or death. **Bovine hyperkeratosis scores are for period animals were in pen. ***Data on these calves cover a 31-week period only.


1
4
2
5
3
6

and these results as well, do not define what score value becomes significant. The score or rating value permits only a very general conception of what has occurred. For example, the average score of 9 calves with bovine hyperkeratosis, during the time they were kept with the test calves in lot 14, was 19.7 (table 1). Seven of these 9 calves died with the disease during the time they were in contact with the test calves. The 8 calves with hyperkeratosis of the skin which were kept in contact with the test calves in lot 13 had an average rating value of 7.0. The 8 calves kept in contact with the test calves of lot 12 had an average rating value of 5.1 (these calves had papilloma of the mouth as the predominating lesion). The 6 calves maintained with the test calves in lot 11 had a score of only 2.7 (these calves could not be considered as being affected with bovine hyperkeratosis). The average bovine hyperkeratosis score for the test calves in lot 11 was 2.4; in lot 12, 1.8; in lot 13, 1.9; in lot 14, 4.0; in lot 16, 3.0; and for the calves at Lincoln, 2.9. Considering individual test calves for the forty-week period of observation, the average scores varied from 0 to 7.3. None of the individual calves had bovine hyperkeratosis scores approaching that of calves definitely sick with the disease. For example, those kept as sick calves in lot 14 had scores ranging from 13.2 to 24.3.

A closer examination of the data becomes necessary in order to appreciate certain differences between the lots of test calves. The symptom of lacrimation was relatively common among all of the test calves during the period of observation. Some calves showed continuous lacrimation from the time they were put on test until the end of the forty-week observation period. Lacrimation as a symptom was somewhat more common in all groups of calves during the period from forty to eighty days after the beginning of the experiment. No one group of calves was more markedly affected than others.

Beginning papillomas of the mouth (fig. 2 and 3) were rather common from forty to eighty days after the beginning of the experiment in the test calves of lots 14, 16, and the group at Lincoln. Among some of these calves, definite papillomas (fig. 1) of the mouth were observed. Four calves of lot 14 showed definite papillomas of the mouth. In 1 of these calves, papillomas of the mouth persisted from fifty-five to 164 days after the beginning of the feeding period. In lot 16, 4 calves had definite papillomas of the mouth. One calf of lot 16 had papillomas of the mouth from forty-three to 133 days after the beginning of the experiment. Seven of the calves in the group at Lincoln had beginning papillomas of the mouth, but none showed definite papillomas at any time.

Sixty days after the start of the experiment, some calves in lots 14, 16, and the group at Lincoln began to develop dermatosis on the dewlap and lower neck. Initially, this was manifest as a dandruff in a mild form. Later, the dandruff was more marked and the epithelium was scaly with thickening of the skin. The texture of the skin remained pliable and did not become tough and leathery as the skin in typical bovine hyperkeratosis. This type of lesion was very mild and transient in 5 of the 15 test calves of lots 11, 12, and 13. The dermatosis was not evaluated in the scoring system.

## Legends for Figures on Opposite Page

Fig. 1—Multiple oral papillomas about left incisor teeth in a case of bovine hyperkeratosis. A large ulcer is evident at the right corner of the dental pad. On the left front corner of the dental pad is a lesion called beginning papilloma.

Fig. 2—Beginning papillomas on gum area behind incisors in a case of bovine hyperkeratosis.

Fig. 3—Papillomas about left incisor teeth and beginning papilloma lesion on gum area behind right incisors.

Fig. 4—Skin area near withers and neck of calf 236 showing hyperkeratosis, loss of hair, and fissures typical of bovine hyperkeratosis. Photograph was taken 404 days after beginning of experiment.

Fig. 5—Calf 134 with hyperkeratosis lesions on skin of neck and shoulders and staining of hair under eye from excessive lacrimation. Photograph was taken 475 days after beginning of experiment and eight days before death.

Fig. 6—Section of liver from calf 236, experimental bovine hyperkeratosis, showing focal "balloon" degeneration of liver cells. x 240.

Definite hyperkeratosis of the skin over the top of the neck, shoulders, and sides of the neck was noted in 2 calves in lot 14, 133 days after the beginning of the experiments. These lesions were not very extensive. Seven calves in lot 16 had mild but definite lesions of the skin typical of bovine hyperkeratosis 164 days after the beginning of the experiment. These lesions continued mild and had disappeared in 4 of the calves at 291 days. Very mild hyperkeratosis was noted in 1 calf of the Lincoln group at 111 days and in another calf 139 days from the beginning of the experiment. These continued mild in character. Two other calves had shown similar lesions by 214 days. By the 273rd day of the experiment, these mild skin lesions were present in only 2 of the calves.

In general, the two lesions of bovine hyperkeratosis, papillomas of the mouth and characteristic skin lesions, were noted in those groups of test calves that were being fed the pelleted supplements suspected of being associated with bovine hyperkeratosis. The test calves maintained in contact with calves from the original outbreak of the disease, and fed prairie hay and soybean meal, did not show definite evidence of bovine hyperkeratosis.

*Results from Additional Feeding of Suspected Pellet Supplement.*—Thirteen test calves were selected that had developed symptoms and lesions of bovine hyperkeratosis to a mild extent as a result of the first course of feeding with suspected pellets. Two calves (51 and 139) of lot 14 were given an additional feeding of suspected pellets. Five other test calves (27, 83, 78, 86, and 110) from lots 14 and 16 were not given the suspected feed but were held for comparison with those that did receive the additional feeding of the suspected pellets. Six calves of the group of 10 at Lincoln were also selected for the investigation of additional feeding of pellets. Calves 48, 236, and 144 received the suspected pellets, and calves 36, 66, and 129 were held as controls. Since the second course of feeding was merely an extension of the original experiment, the entire investigation must be regarded as a single experiment and results from both courses of feeding are presented together.

All of the 5 calves receiving the additional course of feeding with suspected pellets died with the disease. None of the 8 calves receiving a single course of feeding with suspected pellets died.

Dermatosis of the dewlap and neck was

TABLE 2—Time and Duration of Some Lesions of Bovine Hyperkeratosis in Experimental Calves*

| Calf | Highest score and day of exper. noted | Dermatosis of dewlap and neck | Typical hyperkeratosis | Papilloma of mouth | |
|---|---|---|---|---|---|
| | | | | Mild or beginning | Extensive or definite |
| 48 died | 30 @ 357 | 40 to 111** | 214 to 360 | 40 to 84 | ..... |
| 236 died | 22 @ 404 | 60 to 167 | 139 to 404 | ..... | ..... |
| 144 died | 23 @ 483 | 60 to 214 | 357 to 483 | 40 to 60,355,483 | ..... |
| 51 died | 20 @ 395 | 43 to 164 | 133 to 395 | 43 to 133 | ..... |
| 139 died | 20 @ 400 | 43 to 133 | 133 to 400 | 55 to 291 | 76, 164, 305 |
| 36 | 7 @ 60 | 60 to 303 | ..... | 40 to 84 | ..... |
| 66 | 5 @ 214 | 40 to 303 | 167 to 303 | ..... | ..... |
| 129 | 10 @ 111 | 40 to 303 | 111 to 303 | 60 | ..... |
| 27 | 7 @ 105 | 105 to 291 | 164 to 208 | ..... | ..... |
| 83 | 11 @ 208 | 105 to 291 | 208 | 34 to 133 | ..... |
| 78 | 12 @ 76 | 76 to 291 | 164 to 208 | 55 to 76, 164 | 43 to 133 |
| 86 | 10 @ 291 | 76 to 291 | 164 to 411 | ..... | ..... |
| 110 | 8 @ 164 | 55 to 291 | 164 to 291 | 43 to 105 | ..... |

*All calves received suspected feed for the first seventy-four days of the experiment. Calves 48, 144, and 236 received a second course of suspected feed from the 281st to the 335th day of the experiment. Calves 51 and 139 received a second course of suspected feed from the 215th to the 309th day of the experiment.

**40 to 111 indicates that lesions were noted from 40 to 111 days after the beginning of the experiment.

first noted from forty to 105 days after the beginning of the experiment. This condition tended to last longer (up to the 291st to 303rd day of the experiment) in the calves receiving only one course of suspected pellet feeding than in those calves which had two courses of feeding (table 2). Hyperkeratosis of the skin, typical of bovine hyperkeratosis but mild in character, occurred in all but 1 of the 13 calves. Hyperkeratosis of the skin was apparent in most of the calves by the 164th day of the experiment. Hyperkeratosis persisted and became generalized and severe in the 5 calves receiving the additional feeding of suspected pellets. The hyperkeratosis was mild and disappeared in the calves receiving only one course of suspected pellets. Details of these data are given in table 2. Hyperkeratosis of the skin was not noted in calf 144 until late in the experiment (357th day). However, the preceding physical examination was two months prior to this time, and the condition may have developed between the two examinations. Calf 144 was showing evidence of recovery from bovine hyperkeratosis by the 450th day of the experiment. At this time, it was quite anemic (erythrocytes, 3,200,000 per cmm.; hemoglobin, 8.2 Gm. per 100 ml. of blood) but the numbers of leukocytes and body temperature were normal. There was no evidence of secondary infection. The skin lesions were somewhat less severe (fig. 5). Lacrimation was still present. The appetite of the calf was good. On the 483rd day of the experiment, the calf was killed because of marked anemia.

Mild or beginning papillomas of the mouth were observed in 9 of the 13 test calves beginning about the fortieth day of the experiment and lasting for a variable length of time (table 2). Definite papillomas of the mouth occurred in only 2 of the calves. These were recorded for calf 139 on the seventy-sixth, 164th, and 305th days of the experiment. In addition, this calf had shown mild or beginning papillomas of the oral cavity from the fifty-fifth to the 291st day. Definite papillomas were also noted in calf 78 from the forty-third to the 133rd day.

The scores for bovine hyperkeratosis have more significance in this continuation portion of the experiment. The highest scores were generally noted at the time of death of the calves. The scores varied from 17.0 to 30.0 for the 5 calves receiving the additional course of feed. The highest scores for the calves receiving only one course of the suspected feed varied from 5.0 to 12.0. The time at which these high scores were noted varied from the seventy-sixth to the 291st day of the experiment (table 2).

Biopsies of the liver were made on the 6 calves in the Lincoln group on the 350th day of the experiment. There was moderate fatty metamorphosis of the liver cells in all of the biopsy specimens. The liver specimen from calf 236 showed, in addition, mild lymphocytic infiltration in some of the periportal areas. The bile ducts in these infiltrated portal triads were multiple and showed some evidence of mild proliferation. There were no significant differences between the two groups of calves.

*Necropsy Findings on Experimental Calves that Died with Bovine Hyperkeratosis.*—Since details of the necropsy findings on the calves would be repetitious, the data for calf 236 will be given in detail and the findings for calves 48, 51, and 144 in only a brief form.

Calf 236.—On Aug. 9, 1950, the calf was found lying flat on its side and unable to rise. Examination of the blood revealed: hemoglobin, 6.34 Gm. per 100 cc. of blood and 3,200,000 erythrocytes per cubic millimeter of blood. The total number of leukocytes per cubic millimeter of blood was 15,373. The differential count of leukocytes was as follows: lymphocytes, 3,997; neutrophils, 10,839; young neutrophils, 384; monocytes, 115; and eosinophils, 38 per cubic millimeter of blood. Rectal temperature was 100.3 F. Since the animal was obviously near death, it was killed by electrocution. The skin over the upper half of the trunk, the neck and shoulders, in the escutcheon, and over the scrotum was thickened in the manner characteristic for bovine hyperkeratosis. There was partial loss of hair over the neck and shoulders. Hyperkeratosis was very marked in the region of the neck and shoulders and there was both deep and minor fissuring of the keratinized epithelium (fig. 4). The hair below the left eye was stained from lacrimation. Visible mucous membranes were markedly anemic but there was no evidence of icterus. The liver was somewhat enlarged with rounded edges and the color was red-brown. There was a healed scar

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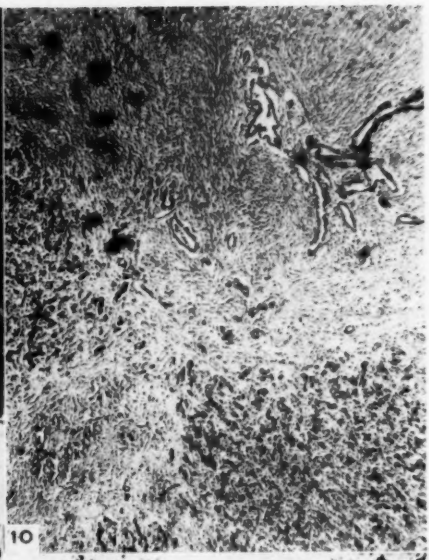

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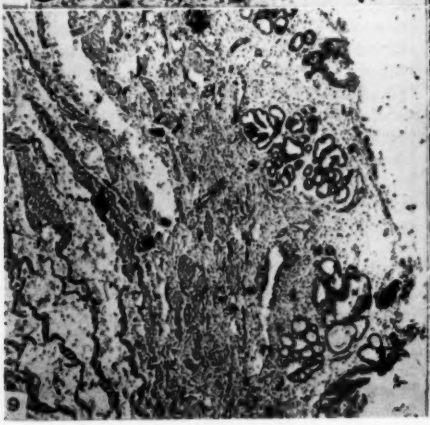

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at the site of the liver biopsy with no adhesions present. The gall bladder was markedly enlarged and contained normal-appearing bile. At the distal end of the gall bladder, there were several large cystic structures (fig. 7) covering an area approximately 5 cm. in diameter. The wall of the gall bladder itself was somewhat thickened and perhaps edematous. One fairly large (1 cm. in diameter) mucous cyst was present in the hepatic duct (fig. 7). The cut surface of the liver was normal except for the rather brownish color. Lymph nodes throughout the viscera were somewhat enlarged and relatively firm. The kidneys were essentially normal. The intestinal tract was examined throughout and was normal. Hydropericardium was present. The descending coronary artery of the left ventricle was surrounded by an area of fibrosis, approximately 2 cm. in diameter. The heart muscle was otherwise normal. The ventral portion of the left thoracic cavity contained a mass of purulent fluid. This occupied about one-half of the left chest cavity and compressed the lung upwards. The right chest cavity contained a moderate excess of clear pleural fluid. A chronic ulcer approximately 2 by 6 cm. was present near the base of the tongue on the left side.

Histology.—In the liver, there were scattered areas of degenerating liver cells. These were focal and involved from one or two cells to approximately 50 in each focus. In these areas, the cytoplasm of the liver cells was represented by a large, clear vacuole (fig. 6). In some of the larger foci, there was mild hemorrhage. There was no definite evidence of hyperplasia of the biliary tree. The wall of the gall bladder was edematous, with the edema extending from the mucosa through the muscularis (fig. 9). The glands below the epithelial lining were dilated and cystic (fig. 8 and 9). There was hyperplasia of the lining cells in some of the cystic glands. In some areas, there was a mild lymphocytic infiltration about some of the glands. A few hyalin casts were found in some of the terminal tubules of the kidney. There was a suggestion of dilation of some of the tubules but this was not particularly marked. The hepatic lymph node showed hyperplasia of the cortex, in which the clearly defined germinal centers were surrounded by masses of lymphocytes. There was a mild fibrosis in the medulla. The lesion about the left coronary artery was a mass of fibrosis with no evidence of active infection. The skin showed marked hyperkeratosis (fig. 11). There was marked irregularity and elongation of the rete pegs (digital prolongations of the epidermis into the corium) and small lymphocytic infiltrations in the papillae, particularly about the smaller arterioles. In some of the papillae, there was mild hemorrhage near the tip. The malpighian layer was thin.

In summary, the characteristic hyperkeratosis of the skin, cystic and edematous condition of the gall bladder and hepatic duct, anemia, and suggested dilation of tubules of the kidney were typical of bovine hyperkeratosis. The empyema was probably directly responsible for the moribund condition of the calf. The leukocytosis of heterophils was no doubt associated with empyema.

Calf 48.—This animal died on a very hot night, June 18, 1950, and the carcass was in poor condition at the time of necropsy. There was definite characteristic hyperkeratosis of the skin over the neck, shoul-

## Legends for Figures on Opposite Page

Fig. 7—Interior exposure of gall bladder and bile ducts with portion of liver from calf 236, experimental bovine hyperkeratosis. Note large cysts in lower portion of gall bladder and thickening of wall. Smaller cysts are also present in hepatic duct.

Fig. 8—Section through hepatic duct of calf 236 (see fig. 7) at location of grossly apparent cyst. Note the dilatation of mucous glands. x 55.

Fig. 9—Section through gall bladder of calf 236 (see fig. 7) at location of large cystic structures. Note dilatation of mucous glands and marked edema in muscularis. x 36.

Fig. 10—Section of liver from calf 51, experimental bovine hyperkeratosis, showing marked cirrhosis and some regeneration of bile ducts. x 55.

Fig. 11—Section of skin from calf 236, experimental bovine hyperkeratosis. Marked hyperkeratosis with elongation of rete pegs (digital prolongations of the epidermis into the corium) is especially evident. x 26.

ders, and back. A papilloma-like lesion was present on the ventral surface of the tongue. There were several ulcers 3 to 4 mm. in diameter in the esophagus. The gastrointestinal tract was examined in its entirety and found essentially normal. The cause of death in this animal could not be determined. However, the hot, sultry weather, together with impaired heat regulation because of the skin lesions, might suggest heat exhaustion as a contributory cause.

Calf 51.—This animal died on July 19, 1950. Corneal abscesses in both eyes had been present for one month. Rapid emaciation was noted for six weeks prior to death, and during the week preceding death the calf had a very poor appetite. The skin of the body was extensively involved with lesions characteristic of bovine hyperkeratosis. There was extensive infection including abscesses of the lungs, empyema, and multiple abscesses of the liver. Histologically, there was marked cirrhosis of the liver with relatively little parenchymatous tissue remaining (fig. 10). There was definite evidence of proliferation of bile ducts in the portal triads. The larger bile ducts were filled with cellular debris and the ducts were surrounded with inflammatory cell infiltration, principally neutrophils. The fibroblastic wall about the abscesses in the liver were poorly developed. The barrier was principally an infiltration of neutrophils. The cause of death of this calf was apparently secondary infection.

Calf 139.—This calf died July 24, 1950. The characteristic hyperkeratosis and thickening of the skin were rather generalized. Numerous ulcers were noted in the mouth, esophagus, and pharynx. There was some localized pneumonia and abscesses in the posterior portion of the lungs. The cause of death of this calf was not apparent from necropsy findings.

Calf 144.—This calf was nearly dead from anemia (erythrocytes, 1,750,000 per cmm.; hemoglobin, 3.57 Gm. per 100 ml. of blood) when killed on Oct. 27, 1950. Hyperkeratosis of the skin was very marked. One small but definite papilloma was present on the hard palate, and several small but distinct papillomas were found in the upper cervical portion of the esophagus. There was a marked enteritis of the small intestine and a very heavy infestation with hookworms (*Bunostomum phlebotomum**). Some nodular worms (*Oesophagostomum radiatum**) were found in the cecum.

## DISCUSSION

The results obtained rather clearly demonstrate the existence of something in the suspected feedstuff that was capable of inciting bovine hyperkeratosis in the test calves. This confirms what previous studies had suggested.[8]** The 15 test calves maintained in contact with calves from the original outbreak of bovine hyperkeratosis in lots 11, 12, and 13 failed to develop significant lesions of bovine hyperkeratosis. This tends to rule out the possibility of contagion or transmission of the disease to susceptible calves. This agrees with reports of Olafson,[7] Gibbons,[2] and others. Furthermore, if contagion were even a partial factor, then the calves in lot 14, which were both fed the suspected feed and maintained in contact with diseased calves, should have had more evidence of bovine hyperkeratosis than the test calves of lot 16. This was not observed.

The development of bovine hyperkeratosis among the test calves maintained in a pen in Lincoln to a degree equal to that noted in the test calves in lots 14 and 16 at Valentine would rule out environment as a factor in the causation of the disease.

One factor not adequately controlled in these trials was the stock of calves used. They were deliberately selected from the same stock as that of calves involved in the original outbreak of the disease. Therefore, the possibility exists that either the stock of calves used was particularly susceptible to the disease or harbored a hypothetical agent which was stimulated by the type of feeding in these experiments. Evidence denying this possibility is suggested by the fact that 3 calves, which were not of the same stock and were on the Valentine premises during the original outbreak, developed bovine hyperkeratosis.[8] These 3 calves had been fed lot 2 pellets.

The results obtained in the experiments described in this report do not completely explain the observations from the original outbreak. In the original outbreak, definite hyperkeratosis was evident and deaths from the disease occurred within three months

*Dr. L. V. Skidmore kindly examined and identified these parasites.

**Since this was written, Olafson and McEntee (Cornell Vet., 44, (1951): 107-109) reported experimental production of bovine hyperkeratosis with a feed concentrate.

from the beginning of the feeding period. In this experiment, hyperkeratosis of the skin was not apparent until after at least five months of the experiment and then only to a mild degree. The first calf died nearly a year after the beginning of the feeding period. One difference between the two groups was the age of the calves at the beginning of the experiment. In the original outbreak, the calves were about 8 months old when first placed on the experimental feedstuffs. In the currently reported experiment, the animals were approximately 15 months old at the beginning of the trials. Several reports and our own observations on field outbreaks of bovine hyperkeratosis indicate the disease to be more common and more severe in animals ranging from 6 to 12 months of age. There may, therefore, be a relation between age and susceptibility to the causative factors of this disease. In another series of experiments, a test calf 4 months old developed lacrimation and skin lesions typical of bovine hyperkeratosis after being fed lot 2 pellets for five weeks. This also suggests the effect of age.

A study of the relationship between period of feeding the suspected feedstuff and the development of the different symptoms was interesting. Certain data are diagrammatically portrayed in fig. 12. The group of 9 calves were survivors of 40 calves in lots 2, 5, 6, and 14. They were fed suspected pellets for 174 days. They were about 8 months old at the beginning of the feeding trial. The other two groups of 5 and 8 animals were 15 months old at the beginning of the feeding trials. In general, lacrimation and papilloma were symptoms that occurred relatively soon after the beginning of feeding and disappeared before the

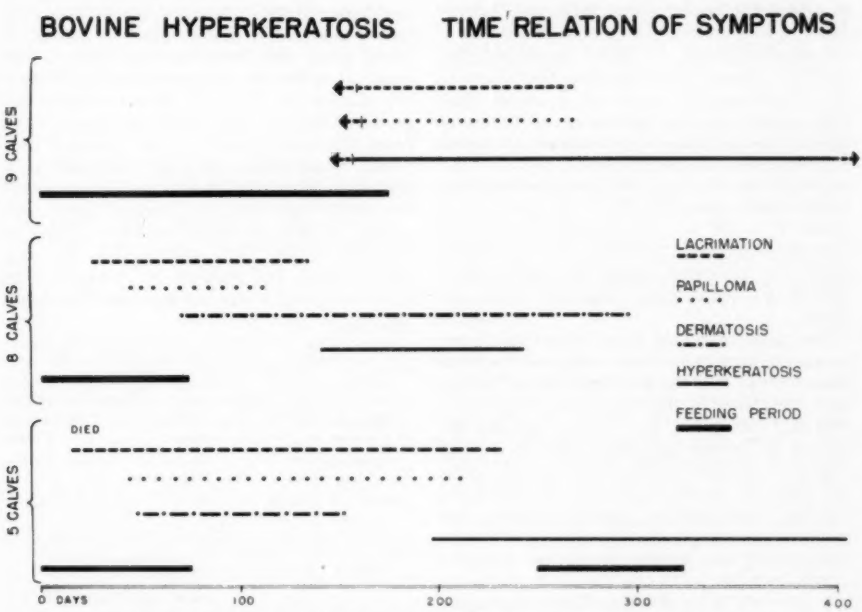


Fig. 12—Diagram of time relationship between symptoms of bovine hyperkeratosis and feeding of suspected feed. End points represent average for the group. Arrows indicate lack of definite end point. The group of 9 calves on suspected feed for 174 days was survivors from the original outbreak of the disease and, while the data do not indicate the beginning of symptoms, the relation to recovery is shown. Hyperkeratosis of the skin was very mild beyond 400 days. The group of 8 calves fed suspected feed for seventy-four days all recovered, and ranges for existence of lesions are shown in table 2. The group of 5 calves given suspected feed for two periods all died. Individual data are given in table 2. The second period of feeding represents the average for the calves of this group.

hyperkeratosis of the skin in those calves that recovered. Among the 13 animals fed for an initial period of seventy-four days, typical hyperkeratosis of the skin was, on the average, not recognized until much later (table 2 and fig. 12). Dermatosis of of the skin existed prior to the hyperkeratosis. In this group of 8 calves, dermatosis of the skin persisted after hyperkeratosis of the skin disappeared. In the 5 calves that died from experimental bovine hyperkeratosis, the dermatosis appeared to blend into the lesion of typical hyperkeratosis of the skin. The average point at which hyperkeratosis became apparent among the 5 calves that died is abnormally high, because 1 calf showed these symptoms at 357 days, thus raising the average. Papillomas lasted, on the average, longer in the 9 calves of the original outbreak and among the 5 calves that died than in the group of 8 calves fed for a short time and recovered from experimental bovine hyperkeratosis. Lacrimation was noted for the shortest time among the group of calves receiving the smallest amount of suspected feed. Lacrimation among the group of 5 calves that died began, on the average, at fourteen days. This low value exists because several of the calves had lacrimation when the experiment was started. Although lacrimation at this time was obviously from some other cause, it could not be distinguished from lacrimation associated with bovine hyperkeratosis. In some calves of this group, lacrimation continued until the time of death.

The demonstration of an association between the particular feedstuffs used in these experiments and the development of bovine hyperkeratosis in the calves receiving these feedstuffs does not help to explain the existence of bovine hyperkeratosis to a mild degree in certain of the calves of the original outbreak. Some of the lots of calves did develop mild bovine hyperkeratosis but received a supplemental feed of a different composition and having no natural feed elements similar to those fed the calves in the current experiments. The reason for these discrepancies may or may not become apparent as more is learned about the cause of bovine hyperkeratosis.

Speculation as to the nature of the factor or factors in the supplemental feed which has been associated with bovine hyperkeratosis would be guess work at this time. Chemical analyses of the suspected feed has provided no conclusive evidence.[8*] Work is now under way in an attempt to learn more concerning the nature of these suspected feedstuffs.

The development of severe parasitism in 1 test calf with bovine hyperkeratosis while 3 other calves not affected with bovine hyperkeratosis in the same pen remained relatively free of the parasites suggests an increased susceptibility to parasitism. An increased susceptibility to bacterial infections associated with bovine hyperkeratosis has been suspected previously.

## SUMMARY

Bovine hyperkeratosis developed in experimental calves after they had been fed a supplemental feedstuff left over from a feeding trial in which bovine hyperkeratosis developed among the calves. The new calves in which the disease was produced were of the same stock but were older than the calves involved in the original outbreak. A longer time and more feedstuff than in the original outbreak was necessary to produce the disease in older test calves. Bovine hyperkeratosis was developed in calves away from the environment of the original outbreak, thus eliminating environment as a factor. Test calves receiving the suspected feedstuff, and also in contact with sick calves, showed no more evidence of bovine hyperkeratosis than calves fed the suspected feed and having no contact with calves affected with bovine hyperkeratosis.

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*Since this was written, McEntee, Hansel, and Olafson (Cornell Vet., *41*, (1951): 237-239) have found a toxic fraction in the ether extract of the feed concentrate investigated by them. Correlation of Wagener's recently published (J.A.V.M.A., *119*, (1951): 133-137) observations associating a wood preservative with hyperkeratosis will require further investigation.

# Relative Sensitivity of Different Skin Areas of Cattle to Intradermal Tests

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*Washington, D. C., and Auburn, Alabama*

JOHNSON,[1] Wadley,[2] and Larsen *et al.*[3] have pointed out that different skin areas of cattle differ in their sensitivity to tuberculin when it is injected intradermally. Areas of high, low, and average sensitivities (neck, back, and sides, respectively) have been designated by these workers. However, numerical values of the relative sensitivities of these areas have not been determined. This paper presents the results of experiments which allow the determination of the numerical values of the following four areas: the back, the upper sides, the lower sides, and the areas starting from about 3 to 4 in. below the top of the neck and extending down and back over the front flank on both sides of the animal and called, in this work, the neck.

## EXPERIMENTAL

Seventy cattle which did not react to intradermal tuberculin and johnin tests were divided into seven groups of 10 each. Two groups were sensitized to heat-killed *Mycobacterium tuberculosis* var. *bovis* by the intraperitoneal route, two groups were similarly sensitized to *Mycobacterium paratuberculosis*, one group was sensitized to *Mycobacterium tuberculosis* var. *hominis*, one group to *Mycobacterium tuberculosis* var. *avium*, and one group to *Mycobacterium phlei*. Groups sensitized to organisms other than the bovine strain of *M. tuberculosis* were included, because these organisms have been considered in connection with no-visible lesion cases.[4]

Quadruplicate tests of four concentrations of each of four materials (three tuberculins and one johnin) were made on each of the 70 cattle, *i.e.*, one replication on each of the four areas mentioned above. Each composite area (corresponding areas on each side of an animal) thus received 16 injections, a total of 1,120 injections per area.

The skin thickness at the site of an injection was measured before the test and again forty-eight hours afterwards with the dermal thickness gauge of Kummer and Johnson.[5]

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## TREATMENT OF DATA

By the use of biometric methods discussed by Wadley,[2, 6] estimates of the relative sensitivities of the four skin areas of an animal were arrived at for each of the seven groups. They are shown in the accompanying table. These estimates depended upon the fact that when the responses from the various dilutions of a material are plotted against the logarithms of the concentrations, a straight line relationship is obtained which permits the estimation of the concentration of the material required to produce a given reaction. The difference between the logs of the concentrations required for each of two materials to produce a given reaction is the log of the ratio of their potencies. This fact permits the estimation of the potency of unknown material in terms of the potency of a standard. Similarly, the sensitivities of various areas of the skin can be estimated in terms of an area which is selected as having a standard sensitivity. In this work, the back region was selected as the standard and assumed to have a sensitivity of unity.

An estimation of relative skin sensitivities of different guinea pigs was made, by methods similar to those described above, by Long and Miles.[7] Their study indicated that thyroxine increased the skin sensitivity of guinea pigs to tuberculin, whereas cortisone or ACTH decreased it.

In studying the data, two alternative procedures were possible, both leading to the same values for the relative sensitivity of the areas for the tests on each group of cattle. One procedure was to compare the sensitivity of the different areas using the results of the four materials separately and averaging the four values obtained for each area. The second was to average the increases in skin thickness of the corresponding concentrations of the four materials on each area and arrive at a single value for the relative sensitivity of the different areas. The latter procedure was selected as being the more convenient.

## DISCUSSION OF RESULTS

The table shows that only in the group designated as *M. paratuberculosis* group 1 was there no significant difference between the back area and any of the other areas. In four of the seven groups of cattle, the

upper side area proved to be significantly more sensitive than the back. In six groups, the lower side was significantly more sensitive than the back and, in two, it was significantly more sensitive than the upper side. In five groups, the neck was significantly more sensitive than the back and the upper side and, in one, it was significantly more sensitive than the lower side.

The average relative sensitivities shown in table 1 indicate a gradual increase from the back down over the sides and to the neck region. The upper side is about 1¾ times as sensitive as the back; the lower side

TABLE 1—Variation in Sensitivity of Different Areas of the Skin of Cattle

| Group | Area | Average reaction in millimeters of different dosages (mg. nitrogen/ml.) | | | | Relative sensitivity | Significance of difference in sensitivity between areas in 2nd column and | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | back | upper side |
| *Mycobacterium tuberculosis* var. *bovis* group 1 | Back | 7.51 mg. N. | 4.62 mg. N. | 2.77 mg. N. | 1.97 mg. N. | 1.0[a] | .. | .. |
| | Upper side | 9.12 | 6.22 | 3.97 | 2.50 | 1.9 | ** | .. |
| | Lower side | 10.41 | 6.53 | 4.66 | 2.83 | 2.6 | ** | .. |
| | Neck | 11.23 | 7.75 | 5.05 | 2.97 | 3.4 | ** | * |
| | | 0.43 mg. N. | 0.21 mg. N. | 0.11 mg. N. | 0.05 mg. N. | | | |
| *M. tuberculosis* var. *bovis* group 2 | Back | 7.77 | 5.44 | 4.41 | 3.59 | 1.0 | .. | .. |
| | Upper side | 9.14 | 6.52 | 5.29 | 3.92 | 1.5 | .. | .. |
| | Lower side | 11.06 | 8.30 | 7.19 | 5.81 | 3.5 | ** | ** |
| | Neck | 12.11 | 8.48 | 6.86 | 5.57 | 3.2 | ** | ** |
| | | 1.69 mg. N. | 0.56 mg. N. | 0.19 mg. N. | 0.06 mg. N. | | | |
| *Mycobacterium paratuberculosis* group 1 | Back | 4.77 | 3.27 | 2.18 | 1.28 | 1.0 | .. | .. |
| | Upper side | 5.54 | 3.95 | 2.46 | 1.46 | 1.5 | .. | .. |
| | Lower side | 5.46 | 3.88 | 2.69 | 1.56 | 1.6 | .. | .. |
| | Neck | 7.04 | 4.43 | 2.43 | 1.53 | 2.0 | .. | .. |
| | | 1.25 mg. N. | 0.62 mg. N. | 0.32 mg. N. | 0.16 mg. N. | | | |
| *M. paratuberculosis* group 2 | Back | 6.69 | 4.99 | 4.00 | 2.65 | 1.0 | .. | .. |
| | Upper side | 8.48 | 6.48 | 4.92 | 3.79 | 1.9 | ** | .. |
| | Lower side | 10.31 | 7.45 | 5.66 | 4.23 | 2.6[b] | ** | .. |
| | Neck | 10.77 | 8.67 | 6.57 | 4.96 | 3.8[b] | ** | ** |
| | | 0.43 mg. N. | 0.21 mg. N. | 0.11 mg. N. | 0.05 mg. N. | | | |
| *Mycobacterium tuberculosis* var. *hominis* | Back | 6.55 | 4.91 | 3.52 | 2.96 | 1.0 | .. | .. |
| | Upper side | 8.00 | 6.18 | 5.26 | 3.91 | 2.0 | ** | .. |
| | Lower side | 10.22 | 7.18 | 6.16 | 4.76 | 3.3 | ** | ** |
| | Neck | 12.10 | 8.73 | 6.87 | 5.10 | 4.4 | ** | ** |
| | | 1.25 mg. N. | 0.62 mg. N. | 0.32 mg. N. | 0.16 mg. N. | | | |
| *Mycobacterium tuberculosis* var. *avium* | Back | 4.92 | 3.40 | 2.80 | 2.17 | 1.0 | .. | .. |
| | Upper side | 5.39 | 4.50 | 3.38 | 2.38 | 1.3 | .. | .. |
| | Lower side | 7.01 | 4.93 | 4.23 | 3.19 | 1.9 | ** | .. |
| | Neck | 7.35 | 5.60 | 4.40 | 3.04 | 2.0 | ** | * |
| | | 2.35 mg. N. | 1.18 mg. N. | 0.59 mg. N. | 0.29 mg. N. | | | |
| *Mycobacterium phlei* | Back | 1.86 | 1.25 | 1.04 | 0.76 | 1.0 | .. | .. |
| | Upper side | 2.52 | 1.79 | 1.28 | 0.88 | 1.8 | * | .. |
| | Lower side | 2.77 | 1.71 | 1.28 | 0.91 | 1.9 | * | .. |
| | Neck | 2.58 | 1.68 | 1.16 | 0.53 | 1.4 | .. | . |
| | | 1.11 mg. N. | 0.50 mg. N. | 0.24 mg. N. | 0.11 mg. N. | | | |
| Average of 7 groups | Back | 5.72 | 3.98 | 2.96 | 2.19 | 1.0 | .. | . |
| | Upper side | 6.88 | 5.09 | 3.75 | 2.69 | 1.7 | * | .. |
| | Lower side | 8.18 | 5.71 | 4.55 | 3.33 | 2.5 | ** | .. |
| | Neck | 9.03 | 6.48 | 4.76 | 3.38 | 2.9 | ** | .. |

*Means that the difference is statistically significant. **Means that the difference is statistically highly significant. [a]In all instances, the black area was considered as the standard. [b]Lower side found to be significantly less sensitive than the neck in this instance.

about 2½ times, and the neck about 2¾ to 3 times as sensitive. Johnson[1] and Larsen *et al.*[3] have reported that the skin of the region of the caudal fold and vulva is less sensitive than that of the back. Although not so many tests could be made on this skin region as on the areas studied in the present work, the evidence of Johnson and Larsen *et al.*, obtained from tests carried out on experimental animals, and in the field, as reported by Swindle *et al.*,[8] indicates that the relative sensitivity of this region must be less than 1.0. It is evident, therefore, that skin tests on the caudal fold or vulva are not so likely to elicit reactions from animals of low sensitivity as are tests on the neck. It is equally evident that tests on the neck would produce more no-visible lesion cases if used routinely in the field. For this reason, cervical testing is reserved for study of problem herds in which repeated testing has failed to clear up a source of infection.

## SUMMARY

Tests of tuberculins and johnin at four levels on each of four areas—the back, upper side, lower side, and neck—on 70 artificially sensitized cattle have furnished data which permitted the estimation of the relative sensitivities of these areas.

Assuming an estimate of 1 for the back, the estimate for the upper side is 1¾; for the lower side, 2½; and the neck, 2¾ to 3.

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Helen S. Bayless, Notary Public (commission expires October, 1953)."

# The Cytological Changes in the Cervical Mucosa of the Cow (Bos Taurus) Throughout the Estrous Cycle

JOHN B. HERRICK, D.V.M., M.S.

*Ames, Iowa*

THE GENERAL adoption of artificial insemination of cattle in the United States has focused attention on the need for greater knowledge of the physiology of reproduction in both sexes of the species *Bos taurus*. The present-day techniques of artificial insemination center around the cervix uteri.

The normal histological picture of the cervical mucosa during the entire estrous cycle of an individual animal has not been previously determined. Furthermore, the available literature fails to show that any attempts have been made to differentiate between mucus-secreting cells and possible other types of epithelial cells present in the mucous membrane of the cervix uteri, nor has an attempt been made to study the cyclical cellular changes in an individual animal.

The purpose of this investigation was to review the literature of histology and anatomy of the cervix and, by histological study, to determine:

1) The type of epithelium in the cervix.
2) The kind of cells that produce mucus.
3) The distribution of mucin-producing cells (so-called goblet cells).
4) The cytological changes which occur in the cervical epithelium throughout the estrous cycle.

Most of the studies of the cervix have been incidental to the entire histological investigations of the reproductive tract as a whole, without detailed study of the cervix alone. These investigations have not been concerned with the cyclical cellular changes in 1 individual but have been a composite of observations of several different animals. Heape,[6] Papanicolaou,[12] Allen,[1] and Long and Evans[9] studied the cycle of experimental animals. Frei and Metzber,[4] Cole,[3] Brown,[2] and Hammond[5] studied the various changes which occur in the genital tract of the cow during the estrous cycle and pregnancy. Iowa workers —Murphy,[10] Zupp,[13] Humman,[7] and McNutt[11]—reported the cellular changes which occurred in the tubular female genitalia of *Bos taurus* during estrus. Due to conflicting terminology and incidence of variation between animals used in these widespread studies, the histological picture of the mucous membrane of the cervix during the entire estrous cycle of an individual cow needs further investigation.

Condensed from a thesis submitted to the graduate faculty of Iowa State College in partial fulfillment of the requirements for the degree of master of science.

From the Department of Dairy Husbandry, Iowa State College, Ames.

## MATERIALS AND METHODS

The cervices from packinghouse specimens were obtained and studied but due to lack of breeding history were not used. Four cows with normal breeding histories were obtained from Iowa State College dairy herd. An instrument was designed to obtain a section of tissue from the posterior and middle cervix of these cows every day of their cycle. A metal vaginal speculum was used to expose the posterior os of the cervix. With the aid of an illuminating headpiece, the instrument was easily guided to the cervix. By opening the jaws of the forceps and with slight pressure forward and closing again, the writer was able to secure a section of the mucous membrane. This technique made it possible to select a female with known breeding history and obtain tissue every day of the cycle.

The fresh tissue from each of the 4 cows studied was immediately placed in 10 per cent formalin solution. After fixing took place for twenty-four hours, the tissue was dehydrated by the dioxane method, infiltrated with paraffin, and embedded. The tissues were sectioned 8 $\mu$ thick, mounted, and dried for twenty-four hours before they were stained.

The staining technique was first that of routine hematoxylin-eosin; later a mucin stain was used. The latter, Mayer's mucicarmine, was used to determine what cells in the mucous membrane of the cervix were capable of producing mucus. The epithelial cells were measured by the use of a filar micrometer and oil-immersion objective.

The cows were examined *per rectum* after the completion of the biopsy work, and no abnormality could be palpated on the cervix, except in 1 Jersey cow. Therefore, the tissue

sections from her cervix are not included in this study.

The results from the tissue studies of estrus and the sixth, fourteenth, seventeenth, and twentieth day of the cycle of cow 2781 are included in this report.

## EXPERIMENTAL RESULTS

*Epithelial Changes in the Cervix Throughout the Estrous Cycle of Holstein-Friesian Cow 2781, Born March 19, 1946.*—Estrus (Fig. 1).—Tissue was removed from the middle portion of the cervix by dilating the posterior os and gently manipulating the instrument into the lumen of the cervix. The cow exhibited no physical signs of heat, but the vaginal walls were moist and glistening, and a small amount of mucus was streaming from the posterior os. The mucous membrane covering the posterior os and the deep part of the cervix was congested. (The cow was observed to be in heat six hours later.) A rectal examination revealed a follicle on the right ovary. Mucus streamed from the vulvar lips following this examination.

The entire lining of the cervix consisted of simple columnar epithelial cells which were well filled with mucus. At the bottom of the primary and secondary folds were crypts in which the columnar epithelium appeared to be pseudostratified, and near the basement membrane in these crypts were many nuclei of small cells. The cells of the cervix were all capable of producing mucus, as determined by a mucus stain. The cells at the apexes of the folds and on the walls of the folds had ruptured at their distal ends, and mucus streamed from them. The nuclei of the cells were elongated and appeared to be moving into the distal portion of the emptying cells. In many cells, the nuclei could not be observed. (The average size of both empty and mucus-distended epithelial cells was 16 by 8 $\mu$.)

The lamina propria was made up of connective tissue fibers, blood vessels, and lymph vessels. Nerve tissue was not observed in this study because of the type of stain used. The fibers of the connective tissue were loosely arranged and contained small, darkly stained nuclei. The lumens of the arterioles and venules were distended. Edema was observed in the lamina propria.

Sixth Day after Estrus (Fig. 2).—The posterior os when viewed through the speculum had the same appearance as it had had the previous day. Fresh tissue was removed from the ventral portion of the posterior os.

Fig. 1—Epithelial changes in the cervix of Holstein-Friesian cow 2781 at estrus. x 257.

The epithelium lining the cervix now was uniform in height and complete; there was little secretion from the cells, but an occa-

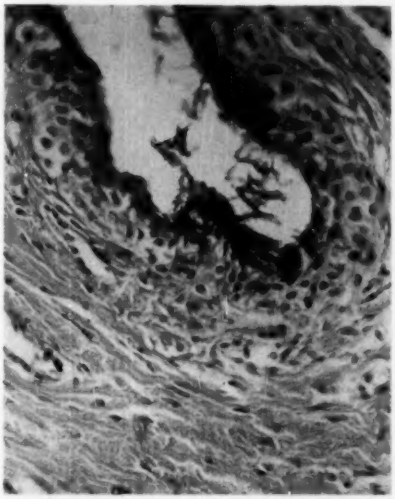

Fig. 2—Epithelial changes in the cervix of Holstein-Friesian cow 2781 six days after estrus. x 257.

sional cell produced mucus which covered the epithelium with a thin film. The nuclei of the epithelial cells took a dark stain, and

Fig. 3—Epithelial changes in the cervix of Holstein-Friesian cow 2781 fourteen days after estrus. x 257.

a large number of nuclei was observed in the base of the crypts. The cells were not as large as they had been in the proestrous

Fig. 4—Epithelial changes in the cervix of Holstein-Friesian cow 2781 seventeen days after estrus. x 257.

stage. (Size of columnar epithelial cells, 12 by 8 $\mu$.)

The connective tissue fibers were thin and compactly arranged in the lamina propria and the nuclei took a dark stain. Some of the nuclei were granular and were situated in large cells close to the basement membrane. Few blood vessels were evident and these appeared small in size.

Fourteenth Day After Estrus (Fig. 3).—The posterior os appeared blanched, dry, and constricted. The tissue was removed from the first annular ring.

The nuclei of the cells of the epithelium in the posterior os appeared to be small, oval to round, and took a dark stain. They were situated near the basement membrane. It was difficult to differentiate the basement membrane from underlying connective tissue strands, even with the silver nitrate impregnation. All of the cells took the mucin stain, an evidence that they all produced mucus. (Size of columnar epithelial cells, 14 by 9 $\mu$.)

The lamina propria was similar to that observed on the twelfth and thirteenth days. The connective tissue fibers were small and compactly arranged. A few vessels situated deep in the lamina propria were visible.

Seventeenth Day After Estrus (Fig. 4).—Follicles were not palpated on either ovary by a rectal examination prior to the tissue removal. The vaginal vault and the posterior os were covered with a thin layer of mucus, but no mucus was observed streaming from the cervical canal.

The simple columnar cells which line the folds in the cervix all stained deeply with the mucus stain. The nuclei of the epithelial cells appeared to be elongated and to be moving into the body of the cell instead of lying close to the basement membrane as they had during the immediately preceding days. Many small cells, which stained lightly, were observed near the basement membrane. (Size of columnar epithelial cells, 17 by 10 $\mu$.)

Twentieth Day After Estrus (Fig. 5).—A rectal examination revealed that one of the follicles on the right ovary had increased in size, whereas the other had not. When viewed through a speculum, the vagina appeared well lubricated with a watery mucus. The walls of the vagina appeared congested. An intense hyperemia was noted in the portio vaginalis and on the posterior os. The posterior os had di-

lated to the extent that the biopsy instrument was easily inserted into the cervical lumen the distance of 1 in.

The epithelial cells which line the cervix had increased in size and height. A slight amount of mucus containing cellular detritus covered the entire epithelium. An occasional epithelial cell was observed secreting mucus. All along the basement membrane border an increase in the number of nuclei was observed, especially in the bottom of the crypts. These nuclei varied in intensity of staining; some appeared to be lightly stained and others took a deep, dark stain. (Size of columnar epithelial cells, 19 by 12 $\mu$.)

The lamina propria was very loosely arranged with the connective tissue fibers some distance apart, the large nuclei of which took a moderate stain. The blood vessels were dilated, and many small vessels were now visible.

## DISCUSSION

The experimental cows 2781, 2976, and 2412 were selected from a large group of females and were under study simultaneously. There was no marked difference in the behavior or in the cervical, histological pictures of the 3 cows. Therefore, 2781 was selected to represent the group. No photomicrographs were made of the cervical tissues of cows 2976 or 2412.

The sections of the cervix were taken primarily from the posterior os with a biopsy instrument. The cervix of the cow is provided with many longitudinal folds and usually four annular folds of mucous membrane, arranged spirally. Throughout the length of these folds, the mucosa is thrown up in secondary, tertiary, and often quaternary folds. These folds are lined with simple columnar, nonciliated, mucus-producing epithelial cells. Each cell constitutes a unicellular gland. Due to the folding of the mucous membrane of the cervix, the epithelium is arranged so that it appears to contain many branched tubular glands. In the base of each fold, there also is epithelium which appears to be stratified, a structure which may be due to the compression of the mucous membrane by the tight sphincter muscle which surrounds the cervix. It is also possible that the oblique cut with the microtome knife at the base of these folds may account for this apparent stratification.

The squamous epithelium of the vagina joins the columnar epithelium of the cervix near the portio vaginalis. No sections were taken which contained stratified squamous epithelium.

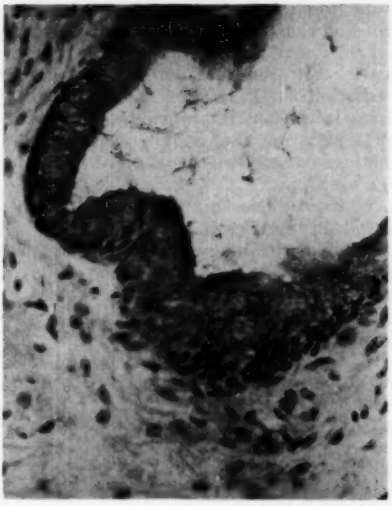

Fig. 5—Epithelial changes in the cervix of Holstein-Friesian cow 2781 twenty days after estrus. x 257.

The estrous cycle was divided into estrus, metestrus, diestrus, and proestrus.

*Estrus.*—Estrus, which lasted twelve hours, consisted of the physical signs of heat, with a large amount of clear, watery mucus flowing from the cervix. Histologically, the cells during this period were loaded with mucus, the majority of them having emptied their secretion at this time. In a large number of these cells, the nuclei were absent. Small cells were also observed near the basement membrane. Although mitosis could not be observed in these sections, the cyclical changes in cell size and staining intensity indicated cell multiplication.

In the lamina propria during the estrus period, there was considerable edema, as evidenced by the increase in size of the vessels in that area.

*Metestrus.*—The loaded stage of the cells observed at estrus had disappeared. Many of the cells had been completely destroyed, and the nucleus and cytoplasm had been

emptied into the cervical lumen. Other cells gave the appearance of having lost only the mucus contents, yet having retained the nucleus. The cells at the distal end of the folds were the first, and those at the base of the crypts the last, to empty their contents into the lumen of the cervix. There still was considerable activity of the epithelial cells near the basement membrane, evidence that regeneration of the mucous membrane had occurred. The connective tissue in the lamina propria was still loosely arranged, but the vessels were collapsed and less congestion was noticed in the entire area.

*Diestrus.*—Diestrus was the quiescent period in the cervix. The epithelial cells were filled with mucus, but these cells were not as high as those observed during estrus. An occasional cell emptied its contents into the lumen of the cervix, so that the epithelial cells were always covered with a small amount of mucus. The nuclei in the epithelial cells were small and took a dark stain.

The lamina propria was very compact. The connective tissue fibers were arranged in tight bundles and little, if any, congestion was noticed. The larger vessels were collapsed and the smaller ones were not distinguishable.

*Proestrus.*—During this stage of the estrous cycle, the cells were in the stage of regeneration. The epithelial cells had increased in size and were filled with mucus.

In the lamina propria, the connective tissue cells had increased in length and contained large nuclei which stained lightly. The vessels had increased in diameter, and there was the appearance of congestion throughout the area.

The physiological loss of cells in the epithelium of the cervix was compensated for by the regeneration of cells near the basement membrane. This regeneration probably was the result of mitotic division of the cells already arranged in the epithelium, although mitosis was not observed. In the denuded areas, these new cells probably arose from adjacent epithelial cells and gradually covered the area, but the new cells did not assume the appearance of columnar epithelial cells for some time.

## CONCLUSIONS

1) Mucus is constantly secreted by the columnar epithelial cells of the cervix but the volume varies with the stage of cycle.

2) All columnar epithelial cells of the cervix produce mucus; therefore, all may become goblet cells.

3) The maximum fullness of mucus-producing cells occurs just at estrus, as shown by the mucicarmine stain.

4) The luminal ends of the cells rupture at estrus and discharge their cytoplasm (mucus) into the lumen of the cervix. The cells on the apexes and sides of the primary, secondary, tertiary, and even the quaternary folds are most likely to empty during estrus, while the cells in the crypts remain filled and empty at irregular times.

5) At each estrus, some cells degenerate, losing their nuclei and cell membranes in the form of cellular debris. The majority of cells, however, retain their nuclei, repair their cell walls, and replenish their cytoplasm prior to the subsequent estrus. During this time, the nuclei move out into the cells away from the basement membrane, become more rounded and oval, and are gradually compressed at the attached end of the cell as the cytoplasm is replenished.

6) The type of cell regeneration to replace the destroyed or degenerated ones is not known, as no mitosis was observed. It is presumed that mitosis does occur to replace the lost cells.

7) During estrus, the intercellular substance increases in amount in the mucosa and submucosa, producing edema and enlargement of the cells, which accounts for the relaxation of the cervix at this time.

8) At the same time, the vascularity of the lamina propria definitely increases, as evidenced by the increased number of vessels as seen in histological preparation taken at estrus. In fact, the increased circulation probably accounts for the relaxation of the cervix and the increase in the number of fibrils making up the basement membrane.

9) There is a definite correlation between the vascularity of the cervix and the character of the mucus secreted during the various stages of the estrous cycle.

10) As the vascularity of the cervix decreases, there is an alteration in the quantity and quality of the mucus secreted.

11) During estrus, the mucus is abundant, clear, adhesive, and contains considerable cellular debris. During metestrus,

the mucus is less abundant, tinged with blood from the uterus, and is adhesive-cohesive in character, containing a moderate amount of cellular debris. During diestrus, mucus is minimal, cohesive, and contains very little cellular debris. In the proestrous stage, there is a gradual increase in mucus secretion which is cohesive-adhesive and a minimal amount of cellular detritus.

12) The average measurements of the cervices of 22 virgin heifers were 2.3 in. in length and 0.75 in. in diameter. The cervices of 24 mature cows gave an average length of 3.4 in. and diameter of 1.6 in.

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# A Clinical Pathological Study of Experimental Leptospirosis of Calves

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ALTHOUGH A NUMBER of reports on bovine leptospirosis in America have been made since 1945,[1, 2, 4-11] an understanding of the fundamental nature of the syndrome has not been forthcoming from observations of field cases. For this reason, leptospiral infections in calves were produced experimentally and were studied by clinical pathological techniques, cultural methods, and direct observations.

## MATERIALS AND METHODS

Six calves were used and are designated as No. 1, 2, 3, 4, 5, and 6 for the purposes of this discussion. Calves 1, 2, 3, and 4 were females, approximately 6 months old, and were bought at public sales. Calves 5 and 6 were males, under 3 months of age, and were procured directly from a nearby farm. All the calves were in good condition and apparently normal, with the exception of No. 5; it was thin, stunted in growth, and not vigorous.

The leptospiral strains used to infect the calves had been isolated by the author from field cases of bovine leptospirosis.[9] One strain, N.Y.A., was isolated from the urine of an adult cow while it was in the acute phase of leptospiral infection. The other strain, N.Y.B., was cultured from the blood of a calf which had died from a fulminating type of leptospirosis. Calf 1 was inoculated with 2 cc. of allantoic fluid from a chicken embryo which had been infected with N.Y.A. strain. This leptospiral culture had undergone 18 serial transfers in chicken embryos. Calves 2, 3, 4, and 5 each were inoculated with N.Y.A. strain by the injection of 2 cc. of fresh, defibrinated blood which had been taken from infected guinea pigs during the febrile stage of the disease. The inoculum used to infect calf 2 was from the eighteenth serial guinea pig passage of the leptospiral strain; that used with calves 3 and 4 from the twenty-fifth passage, and from the twenty-sixth in the case of calf 5. Calf 6 was infected with the N.Y.B. strain which had been maintained in guinea pigs through 25 serial transfers. All inoculations were made by subcutaneous injection in the lateral cervical region.

The calves were kept in isolation units to prevent undesired concurrent infections. Rectal temperatures were taken daily and the animals were observed for signs of disease. Blood was taken from the jugular vein and urine was obtained by manual stimulation of micturition.

The following determinations on the blood were made by standard hematological techniques: erythrocyte count, erythrocyte fragility, total leukocyte count, and differential leukocyte count. The erythrocyte-sedimentation rates of calves 1 and 2 were determined by reading the number of millimeters of fall after eighteen hours' standing in a Wintrobe tube at room temperature and were uncorrected for packed cell volume. With calves 3, 4, 5, and 6, sedimentation rates were measured by the method described by Van Zijl,[12] and were corrected for packed cell volume obtained by the technique of Wintrobe.[13] Hemoglobin concentrations were measured with a Spencer hemoglobinometer. Daily cultures in the medium of Chang[3] and periodic inoculations of guinea pigs and embryonated eggs were made with the blood from the calves. The techniques of cultivation and animal inoculation which were used have been described in a previous publication.[9] Samples of blood were tested routinely by the clinical pathology laboratory of the New York State Veterinary College, Ithaca, for total nonprotein nitrogen, the thymol-turbidity end point, and Van den Bergh's reactions. Daily urine samples were tested for protein content and specific gravity by standard techniques.

At the termination of the experiment, each calf was subjected to autopsy and examined for gross lesions. Preparations were made of affected organs for histopathological examination, and the blood, kidneys, and urine were cultured in artificial medium, guinea pigs, and embryonated eggs to detect resident leptospiral infection.

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The information contained in this report was initially presented to the Graduate School of Cornell University, Ithaca, N. Y., as one section of a doctorate thesis.

At the time this work was performed, the author held a postdoctorate research fellowship awarded by the Division of Research Grants and Fellowships, National Institutes of Health, United States Public Health Service. The latter office gave additional support by means of a grant-in-aid.

At present, the author's address is Rocky Mountain Laboratory, Public Health Service, FSA, Hamilton, Mont.

Acknowledgment is made of the valuable assistance of Dr. C. G. Rickard in the execution of the clinical pathology work and of Dr. Peter Olafson in the interpretation of the histopathological findings.

## RESULTS

The details of the clinical pathology of each case are presented in charts 1 to 6, inclusive. A presentation of the composite findings follows. Fever occurred seven to nine days after inoculation in the calves given guinea pig material. The calf injected with allantoic fluid showed fever on the fourth day. The duration of fevers varied from two to four days, and the peak temperature of all calves except No. 5 lay between 41.3 and 41.8 C. (106 to 107 F.). The peak temperature of calf 5 was 40.7 C. (105 F.). During the fever, the animals became thin and were depressed. The emaciation persisted for two to five weeks, depending on the chronicity of the infection. During the time of fever, a moderate

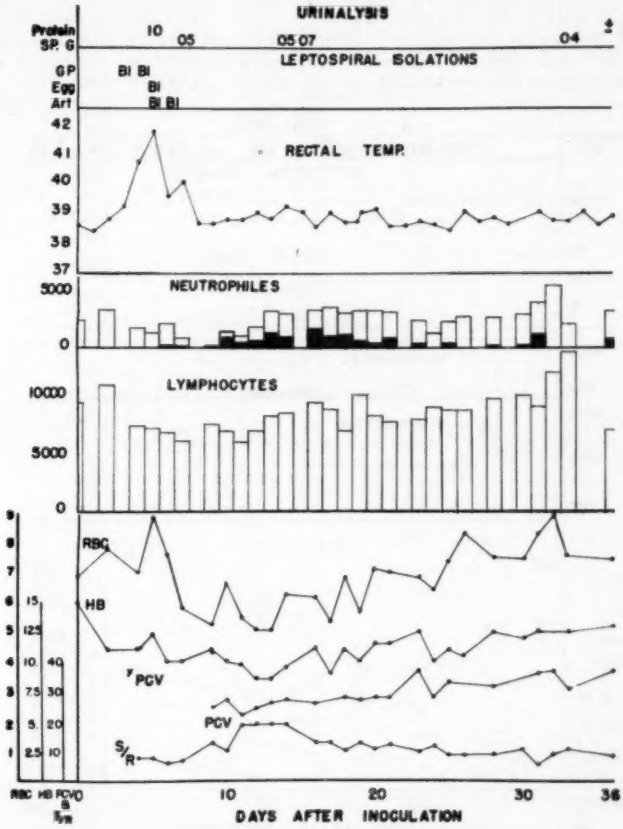



Chart 1—Clinicopathological and cultural features of leptospiral infection calf 1.

*Urinalysis.*—Protein — milligrams per 100 ml. of urine; ± = trace; Sp. G. — last two figures of hydrometric reading, *i.e.*, "10" would be 1.010.

*Leptospiral Isolations.*—GP = Guinea pig isolation; Egg = by embryonated egg culture; Art = by artificial medium culture; Bl = from the blood; U = from the urine.

*Rectal Temperature.*—in degrees centigrade.

*Neutrophils.*—number per cmm. of blood; solid black = number of immature forms.

*Lymphocytes.*—number per cmm. of blood.

*Erythrocyte Factors.*—RBC = number of erythrocytes per cmm. of blood; Hb = hemoglobin concentration, grams per 100 ml. of blood; PCV = packed cell volume in per cent; S/R = for calves 1 and 2: sedimentation rate, uncorrected value, in millimeters per eighteen hours—Wintrobe tube. Calves 3, 4, 5, and 6: mean hourly sedimentation rate, corrected, Van Zijl method.

*Hemoglobinuria.*—line indicates duration.

diarrhea was seen in the older calves and a profuse, watery diarrhea in the younger ones. Fever decreased the vigor of the calves and normal strength did not return while emaciation persisted. At the time of hemoglobinuria in 3 of the calves, the urine became pink, brown, or an opaque black, depending upon the blood pigment concentration in the urine. Calf 5 did not follow the general pattern of physical signs but deteriorated continuously in general condition until it became moribund.

A leukopenia developed which reached its lowest level some time between seven and twelve days after inoculation near or at the time of fever. The differential counts revealed the most significant leukocytic changes. Both lymphopenia and neutropenia developed but were, at times, masked in the total white count because they did not occur simultaneously. The lymphopenia usually reached its lowest level some time between seven and nine days after inoculation. After inoculation, the neutrophil count fluctuated and showed transitory stages of neutrophil increase, which in some cases were definite neutrophilia. But in all cases, a neutropenia developed at some point between the sixth and twelfth day and then gradually remitted.

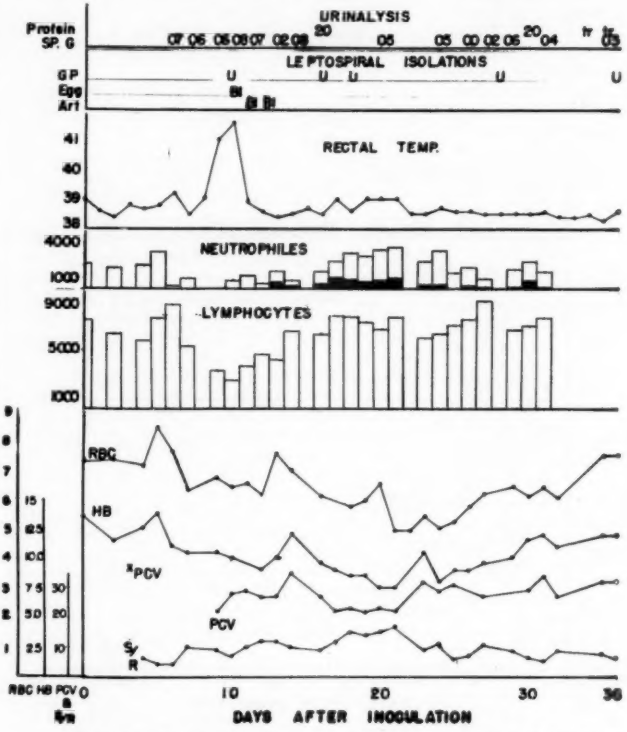



Chart 2—Clinicopathological and cultural features of leptospiral infection, calf 2.

*Urinalysis.*—Protein — milligrams per 100 ml. of urine; ± = trace; Sp. G. — last two figures of hydrometric reading, i.e., "10" would be 1.010.

*Leptospiral Isolations.*—GP = Guinea pig isolation; Egg = by embryonated egg culture; Art = by artificial medium culture; Bl = from the blood; U = from the urine.

*Rectal Temperature.*—in degrees centigrade.

*Neutrophils.*—number per cmm. of blood; solid black = number of immature forms.

*Lymphocytes.*—number per cmm. of blood.

*Erythrocyte Factors.*—RBC = number of erythrocytes per cmm. of blood; Hb = hemoglobin concentration, grams per 100 ml. of blood; PCV = packed cell volume in per cent; S/R = for calves 1 and 2: sedimentation rate, uncorrected value, in millimeters per eighteen hours—Wintrobe tube. Calves 3, 4, 5, and 6: mean hourly sedimentation rate, corrected, Van Zijl method.

*Hemoglobinuria.*—line indicates duration.

Variations in the blood erythrocyte factors were the most significant of the hematological findings. Between the fourth and eighth days, there was a definite trend toward decrease in erythrocyte count, hemoglobin concentration, and packed cell volume. This decrease varied in rapidity and was most marked and abrupt in the cases of calves 3 and 6, where it was associated with hemoglobinuria. As the anemia progressed, there was an increase in immature and aberrant forms of red blood cells. This was more apparent in calves 3, 4, and 6 than in 1 and 2. Polychromatophilia, anisocytosis, poikilocytosis, basophilic stippling, Jolly's bodies, and various types of normoblasts became evident during the stage of greatest anemia and persisted for a week or ten days. Remission of the anemia occurred gradually after the seventeenth or eighteenth day. The sedimentation rate of erythrocytes showed no marked changes, except for a sudden rise and fall after the stage of hemoglobinuria in the cases of calves 3 and 4. A significant finding with calves 3 and 4 was the increased

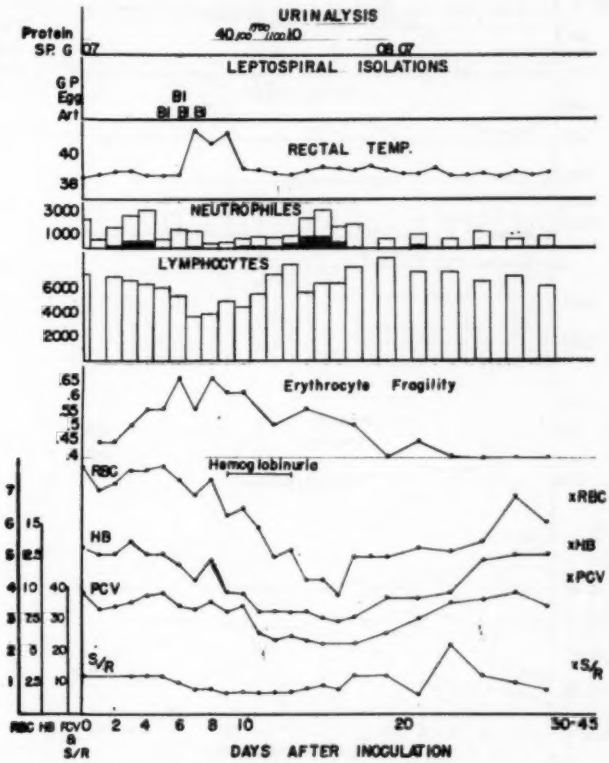



Chart 3—Clinicopathological and cultural features of leptospiral infection, calf 3.

*Urinalysis.*—Protein — milligrams per 100 ml. of urine; ± = trace; Sp. G. — last two figures of hydrometric reading, i.e., "10" would be 1.010.

*Leptospiral Isolations.*—GP = Guinea pig isolation; Egg = by embryonated egg culture; Art = by artificial medium culture; Bl = from the blood; U = from the urine.

*Rectal Temperature.*—in degrees centigrade.

*Neutrophils.*—number per cmm. of blood; solid black = number of immature forms.

*Lymphocytes.*—number per cmm. of blood.

*Erythrocyte Factors.*—RBC = number of erythrocytes per cmm. of blood; Hb = hemoglobin concentration, grams per 100 ml. of blood; PCV = packed cell volume in per cent; S/R = for calves 1 and 2: sedimentation rate, uncorrected value, in millimeters per eighteen hours—Wintrobe tube. Calves 3, 4, 5, and 6: mean hourly sedimentation rate, corrected, Van Zijl method.

*Hemoglobinuria.*—line indicates duration.

erythrocyte fragility during the period of decrease in red cells of the blood.

Nonprotein-nitrogen levels never exceeded normal limits, and were low during most of the syndrome. Van den Bergh's reactions were positive in the indirect phase only in the case of calf 3 during the two days previous to the stage of hemoglobinuria. Thymol-turbidity tests never reached levels indicative of liver damage.

There was albuminuria in calves 2 and 6, the animals that had leptospiruria. Calves 2 and 6 also had a higher incidence of specific gravity values below 1.010. Hemoglobinuria occurred in calves 3, 4, and 6, but not at comparable chronological periods nor to the same degree. In those calves having hemoglobinuria, positive hematin reactions and high protein values were found upon analysis of urine taken in those stages.

Leptospira organisms were cultured from

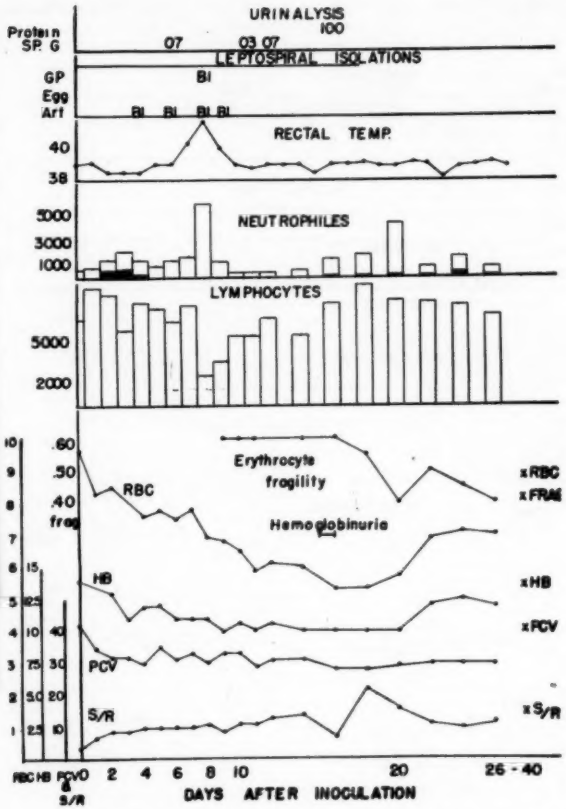



Chart 4—Clinicopathological and cultural features of leptospiral infection, calf 4.

*Urinalysis.*—Protein — milligrams per 100 ml. of urine; ± = trace; Sp. G. — last two figures of hydrometric reading, i.e., "10" would be 1.010.

*Leptospiral Isolations.*—GP = Guinea pig isolation; Egg = by embryonated egg culture; Art = by artificial medium culture; Bl = from the blood; U = from the urine.

*Rectal Temperature.*—in degrees centigrade.

*Neutrophils.*—number per cmm. of blood; solid black = number of immature forms.

*Lymphocytes.*—number per cmm. of blood.

*Erythrocyte Factors.*—RBC = number of erythrocytes per cmm. of blood; Hb = hemoglobin concentration, grams per 100 ml. of blood; PCV = packed cell volume in per cent; S/R = for calves 1 and 2: sedimentation rate, uncorrected value, in millimeters per eighteen hours—Wintrobe tube. Calves 3, 4, 5, and 6: mean hourly sedimentation rate, corrected, Van Zijl method.

*Hemoglobinuria.*—line indicates duration.

the blood by use of artificial mediums, embryonated egg inoculation, and guinea pig inoculation. Leptospiremia was detected in 2 cases before the fever and, in 1 of these, twenty-four hours after inoculation. Leptospira organisms were always present in the blood during the fever and did not persist more than two days beyond the remission of fever. Because of the difficulty of eliminating bacterial contaminants, the detection of leptospiras in the urine by artificial medium and embryonated egg cultivation was unsuccessful, but was shown by guinea pig inoculations in calves 2 and 6. Leptospirurina was present in calf 2, forty-five days after inoculation and in calf 6, twenty-four days. Serums obtained terminally from calves 1, 2, 3, and 4, in 1 : 5,000 dilution, lysed bovine Leptospira. No lysis was obtained with the serums of calves 5 and 6.

At autopsy, all of the calves had, in

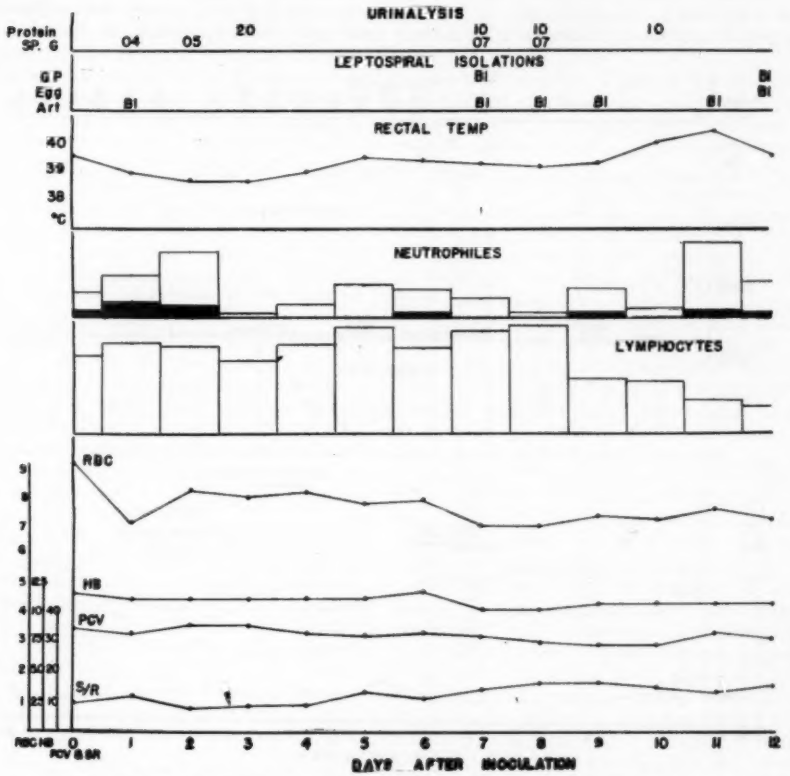



Chart 5—Clinicopathological and cultural features of leptospiral infection, calf 5.

*Urinalysis.*—Protein — milligrams per 100 ml. of urine; ± = trace; Sp. G. — last two figures of hydrometric reading, *i.e.*, "10" would be 1.010.

*Leptospiral Isolations.*—GP = Guinea pig isolation; Egg = by embryonated egg culture; Art = by artificial medium culture; Bl = from the blood; U = from the urine.

*Rectal Temperature.*—in degrees centigrade.

*Neutrophils.*—number per cmm. of blood; solid black = number of immature forms.

*Lymphocytes.*—number per cmm. of blood.

*Erythrocyte Factors.*—RBC = number of erythrocytes per cmm. of blood; Hb = hemoglobin concentration, grams per 100 ml. of blood; PCV = packed cell volume in per cent; S/R = for calves 1 and 2: sedimentation rate, uncorrected value, in millimeters per eighteen hours—Wintrobe tube. Calves 3, 4, 5, and 6: mean hourly sedimentation rate, corrected, Van Zijl method.

*Hemoglobinuria.*—line indicates duration.

varying degrees, grossly observable lesions of interstitial nephritis. These consisted of white foci, visible on the kidney surface, which extended as wedges into the cortex. In those cases which had run a chronic course (No. 1, 2, 3, and 4), the foci were 1 to 2 mm. in diameter on the surface and had distinct margins. In those animals examined at an early stage of the syndrome (No. 5 and 6), the foci were 2 to 5 mm. in diameter on the surface and had diffuse margins. No other visceral lesions were found except in calf 5, which had pale foci several millimeters in diameter in the liver and had advanced serous atrophy of adipose tissue.

In calves 1 and 4, histopathological findings consisted of moderate numbers of areas of interstitial infiltrations in the cortex of the kidney. The infiltrating cells were of the type called polyblastic by some pathologists and by others are termed lymphocytes, plasma cells, or simply round cells. In calf 4, a few plugs of neutrophilic leukocytes were found in some tubules. In the kidneys of animals 5 and 6, there were many small foci of round cell infiltration and plugs of neutrophils in the tubules.

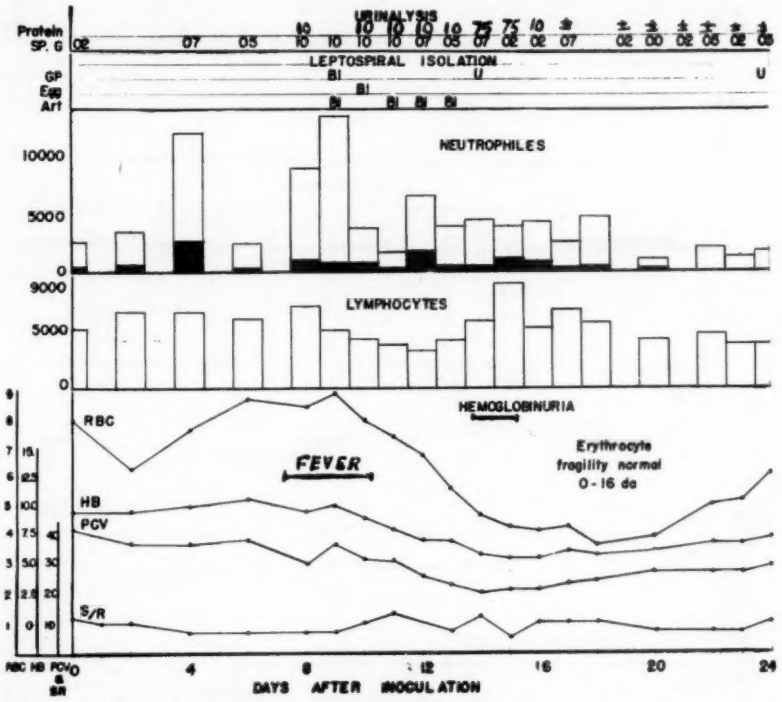



Chart 6—Clinicopathological and cultural features of leptospiral infection, calf 6. The fever line in this particular chart indicates duration of temperatures above 40 C.

*Urinalysis.*—Protein — milligrams per 100 ml. of urine; ± = trace; Sp. G. — last two figures of hydrometric reading, i.e., "10" would be 1.010.

*Leptospiral Isolations.*—GP = Guinea pig isolation; Egg = by embryonated egg culture; Art = by artificial medium culture; Bl = from the blood; U = from the urine.

Line marked fever indicates duration of febrile reaction.

*Neutrophils.*—number per cmm. of blood; solid black = number of immature forms.

*Lymphocytes.*—number per cmm. of blood.

*Erythrocyte Factors.*—RBC = number of erythrocytes per cmm. of blood; Hb = hemoglobin concentration, grams per 100 ml. of blood; PCV = packed cell volume in per cent; S/R = for calves 1 and 2: sedimentation rate, uncorrected value, in millimeters per eighteen hours—Wintrobe tube. Calves 3, 4, 5, and 6: mean hourly sedimentation rate, corrected, Van Zijl method.

*Hemoglobinuria.*—line indicates duration.

Calf 5 showed the accumulation of great amounts of hemosiderin in the phagocytes of the liver and spleen. It also had areas of focal necrosis in the liver. There was some evidence of stimulation of bile duct growth.

Cultures of postmorten material recovered Leptospira from the blood of calf 5 and from the kidneys of calves 2 and 6.

## DISCUSSION

One initial objective of conducting these clinical pathological studies on bovine leptospirosis was to investigate the practicability of certain laboratory tests for the diagnosis of natural cases of the disease, but none was found to be practical. The other aim was to gain insight into the nature of the disease, and this seems to have been reached to a gratifying degree. The experiments were enhanced by the fact that the infections produced were neither fulminating nor inapparent but were of moderate severity. Therefore, they resembled the preponderant portion of cases found in most of the natural enzoötics which thus far have been studied.[2, 5, 6, 9, 10 *]

Because of the unnatural mode of inoculation, it can not be said that the length of natural period of incubation has been established in these experiments. The periods of time between inoculation and onset of fever in these cases agree largely with the incubation periods found by Mathews[8] and by Baker and Little[2] in their experimental infections.

None of the recognized signs of leptospiral infection is entirely pathognomonic. Anorexia, emaciation, transient depression, hypernea, dehydration, hemoglobinuria, and jaundice are physical findings in a number of other diseases. One sign which is closely associated with the syndrome is that which is found in adult, lactating cattle; namely, decreased or interrupted lactation with thickened, yellowish, or bloody milk and a flabby udder.[5] However, in the outbreak of bovine leptospirosis in cows attended by Tierney,[9] the udders were hardened but not inflamed. Baker and Little[2] and Reinhard *et al.*[9] found red or black urine to be a sign in a minority of natural cases. In the experimental infections produced by Baker and Little[1, 2] and in the present work, hemoglobinuria was inconstant in occurrence. Icterus was not observed in any of the animals studied here, but slight icterus could have been missed easily, for all of the subjects had the yellowish integumental pigmentation of the Guernsey breed.

The fact that the signs of leptospirosis are individually not pathognomonic does not leave the field veterinarian without a reliable means of diagnosis. Consideration and integration of all findings in the animals involved in an enzoötic will enable the veterinarian to diagnose this disease with a high degree of accuracy. A more extensive discussion of symptomatology and diagnosis is found in the paper by Little and Baker.[5]

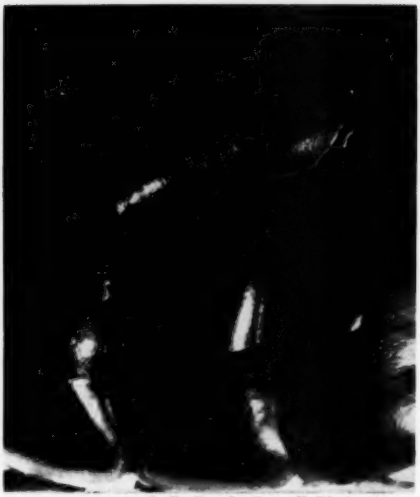
The hematological findings of this study are highly significant. There was always a transitory anemia, which varied in severity from case to case, but which indicated a constant factor of blood destruction. Even in the case of calf 5, which died before frank anemia developed, there was an accumulation of blood pigment in the spleen and liver, indicating excessive erythrocyte destruction. Therefore, the syndrome of bovine leptospirosis can be classed with the infectious hemolytic anemias.

Another hematological feature of the disease is the occurrence of a leukopenia involving both neutrophils and lymphocytes. This response usually occurs concurrently with fever or soon after. In this manner, leptospirosis resembles some viral infections of animals. In a hemolytic, fulminating case, found in a natural enzoötic, a leukocytosis with neutrophilia has been described.[9] There are no reports of the experimental duplication of this feature.

In none of the calves studied did blood chemical findings show the occurrence of uremia. In addition, there was no chemical indication of liver damage, despite histopathological evidence to the contrary. For full substantiation of these findings, the chemical blood tests should be tried on more severe infections than these calves presented.

*Most enzoötics thus far reported have been in dairy herds where the number of exposed animals was limited and the proportion of severe or fatal cases showing hemoglobinuria was small. However, H. C. Smith, in writing "Progress Report on Idiopathic Hemoglobinuria in Oklahoma Cattle," J.A.V.M.A., *102*, (1943): 352, describes a hemoglobinuria in Oklahoma cattle which might well have been of leptospiral origin and which was marked by high morbidity and mortality. All of the animals showing hemoglobinuria died and, in one outbreak, 24 out of 77 calves died. It is reasonable to expect that the successive serial transfers effected by an enzoötic sweeping through large cattle populations, such as are found in western beef herds, would increase the virulence of the disease and thus increase the proportion of acute cases ending in hemoglobinuria and death.

Of the 6 animals used, the 4 which were 6 months of age or older developed significant blood antibody titers against Leptospira. The 2 which were less than 3 months of age did not yield antibodies demonstrable by the lysis technique; however, one of these infections had run a short, fatal course of twelve days. In a group of 10 young calves previously studied by the author,[9] which became infected when less than 3 months old, only 1 developed demonstrable blood antibody titers, and that only to a dilution of 1 : 50. If these observations are borne out in future experience, they will pose a problem in epidemiological and diagnostic serological work in bovine leptospirosis.



—*J. F. Brock, Cornell Photoscience*

**Fig. 1—Gross lesions, kidney, calf 3, forty-five days after inoculation. Note discrete white foci, typical of chronic state.**

—*J. F. Brock, Cornell Photoscience*

**Fig. 2—Gross lesions, kidney calf 5, twelve days after inoculation. Note indistinct margins of foci, typical of acute stage.**

In the examination of the urine, discounting the periods of hemoglobinuria, coagulable protein was detected only in calves 2 and 6, which had leptospiruria, and in calf 5, which died after a short course. The albuminuria was not severe in any case. Also, as reported previously,[9] consistent low specific gravity readings were associated with leptospiral infection but, in the present study, only in those calves which had leptospiruria. Therefore, it can not be said that albuminuria and low specific gravity of urine are always present in cases of leptospirosis.

In the examination of the animal for the presence of Leptospira organisms, the better sources of inoculums for cultivation of the agent are the blood and the blood-filtering organs (kidney, spleen, and liver) during the time of fever. In the chronic stage of the disease, the kidneys are more likely sites of leptospiral foci. Therefore, these organs and the urine may serve as sources of leptospiral cultures. When bacterial contamination is not present, both embryonated chicken eggs and artificial mediums can serve as means of cultivation. Because bovine Leptospira are quickly killed in artificial culture by bacterial contaminants, animal inoculation has been found to be the only reliable means of cultivation of organs, blood, or urine which have not been taken with aseptic technique.

Although focal interstitial nephritis is a constant finding in bovine leptospirosis, sometimes as long as sixty days after inoculation,[2] there is doubt that it is indicative of the presence of Leptospira. Five calves were autopsied fifteen to forty days after the stage of fever. Only 2 had Leptospira in the kidneys as shown by cultivation in guinea pigs, eggs, and artificial mediums, although all had lesions of focal interstitial nephritis. It is possible that the focal infiltrations are stimulated by the

presence of Leptospira and persist after the latter are gone. It is also possible that focal infiltrations are caused by a factor other than the presence of the agent.

These experiments show that there are at least three phases of leptospiral infection in cattle. One is the septicemic phase marked by fever and leptospiremia. Another phase is that of hemolytic anemia. A third phase is that of nephritis which may be accompanied by leptospiruria and the carrier state. It is the author's concept that the variability in the signs of natural cases of bovine leptospirosis is determined largely by variation in the degree of the hemolytic anemia. In peracute cases, the massive blood destruction leads to sudden death, due to insufficient oxygen transfer. Hemoglobinuria is probably caused by the inability of the liver and spleen to cope with the quantity of the products of excessive blood destruction. In addition, it is entirely likely, but not at all proved, that toxic factors are involved in leptospirosis, for the infected animals are often severely affected physically to a degree that seems disproportionate to the grade of anemia or anorexia involved.

## SUMMARY

1) Six calves were inoculated with Leptospira organisms obtained from natural cases of bovine leptospirosis, and cultural, hematological, and clinical studies were made on these animals. The following features of the disease were found:

*a*) A fever—which started as early as the fourth and as late as the ninth day and which lasted two to four days.

*b*) Leptospiremia—which was detectable in some cases before the febrile period and which ended in all cases soon after the end of fever. In 1 calf that died, leptospiremia continued into the moribund state of the animal.

*c*) In 2 cases, leptospiruria occurred after the fever, in 1 as long as six weeks, and Leptospira were isolated from the kidneys of both animals at autopsy. Albuminuria was observed in conjunction with leptospiruria.

*d*) Neutropenia and lymphopenia occurred during or close to the time of fever.

*e*) A transitory anemia occurred in all cases except the short-termed fatal case. In 3 cases, hemoglobinuria occurred in association with erythrocyte decrease. Associated with the progress of anemia in 2 cases was a great increase in red cell fragility.

*f*) In all cases, there were gross and microscopic lesions of focal interstitial nephritis. In 1 calf that died in the acute stage of the disease, focal necrosis was found in the liver.

2) The significance of these findings is discussed in relation to the fundamental nature of the disease and the observable physical signs.

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# Studies of Electrolytes in Body Fluids of Dairy Cattle
# II. Effects of Estrogen on Electrolyte Levels in Body Fluids in Late Pregnancy

A. F. SELLERS, V.M.D., M.Sc., Ph.D., and M. H. ROEPKE, Ph.D.

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THE TENDENCY in pregnant and postparturient dairy cattle toward disorders characterized by changes in the concentrations of several of the mineral constituents of blood has been recognized. The apparent adrenocorticomimetic effects of estrogen[5, 9, 17, 19, 26, 27, 28] suggested the desirability of trying large doses in late pregnancy, since, in mares[6] and in women,[8, 30] estrogen is excreted in large quantity in the latter part of pregnancy, and a sudden fall just before parturition conceivably could be a factor in initiating electrolyte imbalance.

Detailed information regarding blood and tissue levels of estrogen in pregnant and parturient cattle is not presently available. The urinary excretion of estrogen by pregnant cows is relatively low compared to mares and women.[16, 20, 29] Asdell[1] and Cowie[10] have reviewed the somewhat conflicting observations of various investigators. Estrogen has been found in the feces[18] and gall bladder bile[23] of pregnant cows.

## METHODS

Five nonlactating adult Holstein-Friesian cattle were continuously confined in box stalls during the last two to four weeks of pregnancy and for one to two weeks after parturition. Feed consumption was observed and recorded.

A large dose (75 to 100 mg.) of diethylstilbestrol in peanut oil was administered intramuscularly in three or four divided doses over a two-day period.

Three preinjection blood and urine specimens (not quantitatively collected) were taken on successive days, the last being just prior to the first dose of estrogen. Starting on the second day of estrogen administration or the first postestrogen day, samples were collected daily for three or four days, then less frequently to the time of parturition. Starting with the day of parturition, samples were collected daily for three days, then less frequently for approximately one week.

Animal 659 (table 1, fig. 1 and 2) received additional estrogen in graded dosage for seven days preceding parturition; on the day of parturition and four days thereafter, 40 mg. of estrogen were given daily, in two equal doses morning and evening.

Calcium, magnesium, and inorganic phosphate were determined on serum specimens; chloride, sodium, and potassium, on heparinized plasma specimens collected in an oiled syringe, discharged under oil, and centrifuged without delay. This procedure, in the case of the latter determinations, was adopted in view of the necessity for detection of small variations in concentration. Changes in sodium and chloride concentration because of changes in the blood-gas content and in potassium because of diffusion from the red cells attendant upon the long standing necessary for clotting in bovine blood were thus avoided. The amount of heparin used was kept constant. Centrifugations in all cases were carried out after stoppering to prevent evaporation.

In the serum specimens, total calcium was determined by the Clark-Collip[7] modification of the Kramer-Tisdall method. Magnesium was determined by the method of Denis[11] and the Fiske-Subba Row[12] phosphate method was used to estimate the amount of magnesium ammonium phosphate present. The latter method was also used to determine inorganic phosphate.

Chloride concentration in the plasma specimens was determined by the method of Schales and Schales.[24] Sodium and potassium determinations were made flame photometrically[2, 15, 22] on trichloracetic acid filtrates of plasma, using the internal standard method.[3] Sodium interference was added to the potassium standards and potassium interference added to the sodium standards, in amounts corresponding with maximum concentrations of the respective interfering ion which could be present at the dilutions used.[21] A concentration of 10 mEq. per liter of lithium nitrate was employed in both the standards and un-

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knowns. Trichloracetic acid was added to the standards in amounts corresponding with the amounts of this substance present in the plasma filtrates.

Levels of all blood constituents determined were calculated and recorded as milliequivalents per liter of serum or plasma. Phosphate was expressed as phosphorus.

Determinations of the corresponding substances in urine were performed by the methods previously described.[25]

## RESULTS

A small number of animals was used in this study. This fact must be kept in mind when evaluating the results.

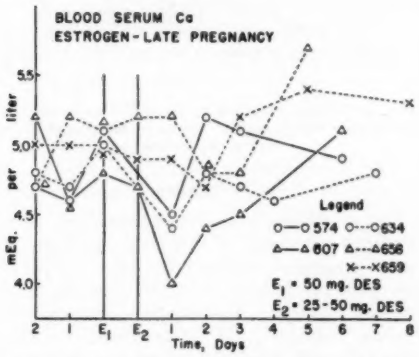


Fig. 1—Levels of total blood serum calcium in dairy cows given large doses of diethylstilbestrol in late pregnancy. $E_1$, $E_2$ = first and second days of estrogen administration; DES = diethylstilbestrol.

Table 1 summarizes the results of studies of blood-electrolyte levels following estrogen administration in late pregnancy and over the parturition period in 5 animals. One animal (No. 633) was followed over the parturition period only. Animal 659 received estrogen over the parturition period, as explained previously.

All values given are averages of two-day cross sections of the family of curves, one such cross section being taken before and one similar cross section after a given effect was studied. This procedure was used in order to take into account day-to-day variations in the level of a given constituent, in so far as possible with the type of effect being studied. A two-day cross section seemed advisable for summary purposes, inasmuch as a given effect rarely lasted more than that length of time. As a matter of fact, such two-day averages tend to mask the magnitude of the greatest rise or fall in a given element because these rises and falls were usually most marked on one day only (fig. 1 and 2).

Cross sections consisted of averaging two successive values for a given constituent; for a group of 5 animals, ten analytical values were averaged to obtain a given figure for table 1. "After" values are two-day cross sections of the family of curves at their highest or lowest points, respectively, when change occurred.

Referring to table 1, it will be noted that the most noticeable changes were falls in total blood serum calcium and serum inorganic phosphate at the time of parturition, as noted by others.

In some individuals (fig. 1), a slight fall in total serum calcium was noted following estrogen administration, with but little change in the group average (table 1).

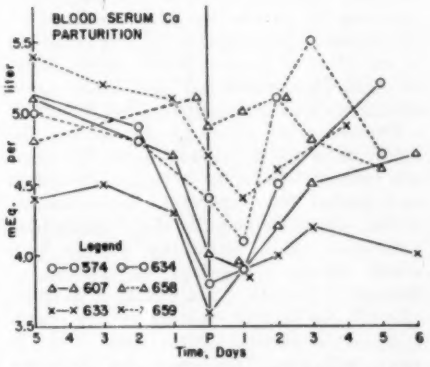


Fig. 2—Levels of total blood serum calcium in dairy cows at parturition, showing values attained at this time for comparison with those previously attained following estrogen (fig. 1). Animal 659 received estrogen continuously over the parturition period. P = day of parturition.

Occasional individual instances of rise in serum magnesium and inorganic phosphate following estrogen were seen. Plasma sodium, potassium, and chloride showed no consistent changes following estrogen.

When the animals were followed through the parturition period, in addition to the fall in total serum calcium and serum inorganic phosphate mentioned previously, a slight rise in serum magnesium was seen

and a slight downward trend of plasma chloride following parturition. Plasma-sodium and potassium levels showed no consistent change over the parturition period.

One animal (No. 659), which had shown a fair serum-calcium response to estrogen administration and withdrawal in late pregnancy (fig. 1), was given graded estrogen dosage starting seven days prior to parturition, beginning with 5 mg. and attaining 40 mg. by the day of parturition. Forty milligrams of estrogen daily were given for four days following parturition. It will be noted in table 1 and fig. 2 that the same type of calcium fall at parturition was experiencd by this animal as by the other untreated animals. This animal also showed a slight rise in serum magnesium and fall in serum inorganic phosphate at the time of parturition (table 1).

Attention was directed, in the case of the urine specimens collected prior to, during, and following estrogen administration, and over the parturition period with which the former was compared, to large changes in excretion of the substances under study. It must be remembered that these urine specimens were not quantitatively collected.

Concentration values of the various elements were plotted against time. Ratios of one element to another were also calculated and plotted for inspection. Particular interest was attached to the magnesium: potassium ratio, since any change here would appear necessarily to be due to change in excretion and not to a diuresis effect.[25] No regular changes in concentrations or in element ratios were noted in most instances. This was true following estrogen administration as well as over the parturition period. The plotted magnesium: potassium ratios seemed fairly regular from animal to animal but showed no marked upward or downward tendency over the parturition period (fig. 3).

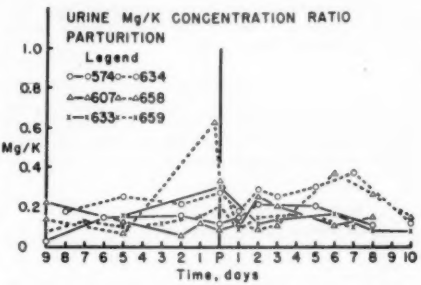


Fig. 3—Urinary magnesium: potassium concentration ratios over the parturition period in dairy cows. P = day of parturition.

## DISCUSSION

Administration and withdrawal of relatively large doses of synthetic estrogen in late pregnancy seemed in some cases to be associated with a slight fall in total serum calcium. The finding of lowered serum calcium is in accord with the observation of Frei and Emmerson[14] using natural estrogen on a nonpregnant animal and that of Folley[13] in lactating animals.

The more consistent and more marked fall in total serum calcium seen at parturition was likewise experienced by a single animal (No. 659) given estrogen continuously over the parturition period in an effort to prevent estrogen withdrawal ef-

TABLE 1—Blood-Electrolyte Levels in Dairy Cattle Given Estrogen in Late Pregnancy and Levels Observed Over the Parturition Period

| | | Blood-electrolyte levels mEq./liter* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total blood serum calcium | | Serum magnesium | | Plasma sodium | | Plasma potassium | | Plasma chloride | | Serum inorganic phosphate | |
| No. of animals | Effect studied | Before | After | Before | After | Before | After | Before | After | Before | After | Before | After |
| 5 | Estrogen | 4.9 | 4.7 | 1.8 | 2.0 | 144 | 143 | 4.0 | 4.3 | 102 | 103 | 9.4 | 9.6 |
| 1 | Estrogen + parturition | 5.2 | 4.5 | 1.9 | 2.1 | 145 | 144 | 4.0 | 3.7 | 109 | 100 | 7.9 | 4.0 |
| 5 | Parturition | 4.8 | 4.2 | 2.2 | 2.4 | 145 | 146 | 4.1 | 4.0 | 106 | 101 | 9.1 | 6.9 |

*All values given are averages of two-day cross sections of the family of curves, taken before and after a given effect was studied. That is, for 5 animals in a group, ten analytical values were averaged to obtain a given figure for the table above. "After" values are two-day cross sections of the curves at their highest or lowest points, respectively, when change occurred.

fects, if such were a provocative factor in total serum-calcium fall at this time.

Blosser and Smith,[4] in quantitatively collected urine specimens, noted a slight increase in urinary magnesium excretion in normally-freshening dairy cows, and a definite decrease in urinary magnesium in cows later developing symptoms of milk fever.

## SUMMARY

1) In a small number of dairy cows given large doses of diethylstilbestrol in late pregnancy, and later followed over the parturition period, blood levels of calcium, magnesium, sodium, potassium, chloride, and inorganic phosphate were studied. The most noticeable changes were fall in total blood serum calcium and serum inorganic phosphate at the time of parturition.

2) In some individuals, a slight fall in total serum calcium was noted following estrogen administration.

3) No appreciable or consistent changes were noted in blood levels of sodium, potassium, magnesium, or chloride following either estrogen administration or parturition.

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# Studies of Electrolytes in Body Fluids of Dairy Cattle
# III. Effects of Potassium on Electrolyte Levels in Body Fluids in Midlactation

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LOWERED serum-magnesium and -calcium levels were described in cases of tetany apparently independent of parturition, in cattle soon after feeding on young and quick-growing grass, by Sjollema and Seekles,[23] and Sjollema.[24] The latter pointed out the abnormality of young grass as regards its high potassium : sodium ratio.[25,26] The condition became known as grass or lactation tetany. Harbaugh and Dennis[7,11] pointed out a tendency toward rise in the blood-potassium : calcium ratio associated with grazing of winter wheat and an even higher ratio when symptoms of tetany were present.

It was desired to learn something of the effects of high potassium given to lactating cattle, in possibly initiating imbalances of other electrolytes. Similar doses of potassium given alone and preceded by biotin,* and high sodium intake were compared. Smith[28] found that in the progressive paralysis of dogs on biotin-deficient diets, either potassium or biotin was effective in treatment. She suggested that perhaps potassium is mobilized temporarily by biotin. Wiese *et al.*[31] produced a posterior paralysis in calves fed a synthetic ration, which was curable by biotin. Flipse *et al.*[9] produced progressive paralysis in calves on synthetic milk rations, which was curable either by potassium or biotin administration.

## METHODS

Five adult Holstein-Friesian cattle in midlactation (4 to 5 months), 4 of which were one to four months pregnant, and one nonpregnant animal (No. 633) were used. These animals were confined in box stalls for several days preceding study, and the feed consumption measured and recorded. Water diuresis was induced as previously described.[21] One to five days later, 0.5 Gm. of potassium chloride per pound of body weight, in approximately 10 gal. of tap water, was introduced into the terminal portion of the esophagus, according to the method of Dennis and Harbaugh,[7] over a period of approximately forty-five to fifty minutes. A control, two-hour quantitative collection of urine was made and a preinjection blood sample collected.

Ten minutes following the end of the injection, a blood sample was taken and another at thirty minutes. A two-hour quantitative collection of urine was made, starting with the beginning of the injection. Blood samples were also collected the following morning.

After a lapse of five days, 2 of the same animals (No. 633 and 658) were given 0.5 Gm. of potassium chloride per pound of body weight following injection of 1 $\mu$g. of biotin per pound of body weight, intramuscularly, on each of two preceding days and again on the morning of the experiment.

A further experiment on another 2 of the same animals that received potassium alone (No. 756 and 759) consisted of administering 0.5 Gm. of sodium chloride per pound of body weight. These trials were carried out two and five days, respectively, following potassium. Sampling was done as before.

One of the potassium animals (No. 781) received 0.65 Gm. of potassium chloride per pound of body weight six days following the first trial, with sampling being performed as before.

The animals were followed electrocardiographically in three of the potassium trials, one potassium-biotin trial, and in the two sodium trials. Leads I, II, III, and a precordial lead were used.

Collection and handling of blood and urine specimens, and their subsequent analysis, were as previously described.[21, 22]

## RESULTS

Plasma potassium showed a prompt rise in 4 of 5 animals given potassium chloride orally; comparable rise occurred in both of

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*Crystalline synthetic B—biotin ($C_{10}H_{16}O_3N_2S$) kindly supplied us by Dr. D. F. Green, Merck and Co., Inc., Rahway, N. J.

2 of the same animals when the potassium chloride was preceded by biotin administration. Smaller rises in plasma potassium occurred when sodium chloride was given (fig. 1).

Plasma sodium rose in all animals given potassium chloride alone, potassium chloride with biotin, and sodium chloride. Elevations of plasma sodium when potassium was given were at least as great as when sodium itself was administered (fig. 2).

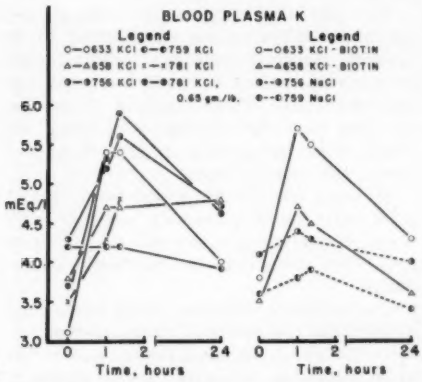


Fig. 1—Levels of plasma potassium attained with KCl administration (left side), and with KCl-biotin and NaCl administration, respectively (right side).

Serum inorganic phosphate rose in all instances of potassium and sodium administration, and in one of two potassium-biotin trials (fig. 3).

Total serum-calcium and serum-magnesium levels showed no regular change, with any of the three trial procedures used. Plasma chloride values rose comparably in all trials.

In 2 animals (No. 756 and 759), blood levels of the various substances under study showed no marked change when water alone was administered; a tendency toward a slight fall in sodium, potassium, and chloride seemed to be present with this procedure.

Changes in absolute renal excretion of the several elements (table 1) seemed interesting. Absolute potassium excretion did not seem to rise as much as might be expected in a two-hour period following potassium or potassium-biotin administration. However, absolute excretion of sodium in the urine when potassium was given showed an increase in 3 animals (No. 633, 759, and 781 with a 0.65-Gm./lb. dose) comparable to that attained when sodium itself was administered and greater than would be expected from a "washing out" effect of water administration.[21] This is noted in conjunction with the observation cited previously that plasma sodium showed a regular rise with potassium administration.

Absolute magnesium excretion, in the three trial procedures of potassium alone, potassium-biotin, and sodium administration seemed to remain essentially constant, as it had with water diuresis,[21] the concentration being inversely proportional to the volume of urine.

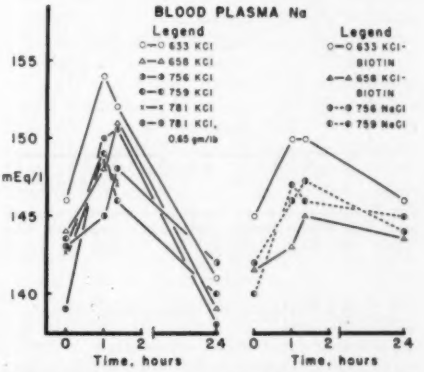


Fig. 2—Levels of plasma sodium attained with KCl administration (left side), and with KCl-biotin and NaCl administration, respectively (right side).

Absolute urinary calcium excretion in the various trials seemed increased to a degree comparable to that expected with water alone. Absolute chloride excretion seemed somewhat increased, in general, as would be expected with chloride administration. Absolute total phosphorus change seemed comparable to that seen with water diuresis.

It will be noted from table 2, showing absolute excretions of the substances studied and calculated as ratios before and during administration, that the potassium : sodium ratio invariably decreased, whether potassium, potassium plus biotin, or sodium be given. This would tend to indicate that the increase in absolute sodium excretion when potassium was given occurred more

promptly or to a greater degree than an increase in absolute potassium excretion, in the two-hour period during and following potassium administration. Further, the outpouring of sodium when potassium was given was quite comparable in degree to that seen when sodium itself was given.

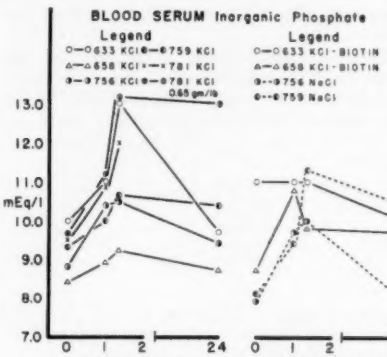


Fig. 3—Levels of serum inorganic phosphate attained with KCl administration (left side), and with KCl-biotin and NaCl administration, respectively (right side).

This latter point gains validity from the observation that the reciprocal change, *i.e.*, increased potassium excretion when sodium was given, could not have been marked. An inspection of table 1 will show that the trials where sodium was given showed increased absolute potassium excretion no greater in order of magnitude than when potassium was given.

The absolute magnesium : sodium and calcium : sodium ratios also tended to be lowered markedly, and the sodium : phosphorus ratio raised markedly by potassium administration. These changes all emphasize the fact that sodium was being excreted when potassium or potassium plus biotin was administered.

Whereas the absolute magnesium : potassium ratio tends to remain constant with water diuresis,[21] it is lowered slightly with potassium administration, as would be expected.

The absolute calcium : magnesium and magnesium : phosphorus ratios seemed affected to about the same degree and in the same direction as with water diuresis; washing out of calcium and of phosphorus would appear to be of about the same mag-

TABLE I—Change in Absolute Renal Excretion of Several Elements with KCl, KCl-Biotin, and NaCl Administration

| Animal (No.) | Expt | Specific gravity* | Ave. vol./hr., liters | Calcium Conc., mEq./L. | Calcium ΔE** | Magnesium Conc., mEq./L. | Magnesium ΔE | Sodium Conc., mEq./L. | Sodium ΔE | Potassium Conc., mEq./L. | Potassium ΔE | Chloride Conc., mEq./L. | Chloride ΔE | Total phosphate Conc., mEq./L. | Total phosphate ΔE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 781 | KCl, | 1.009 | 2.70 | 0.12 | | 5.0 | | 32.00 | | 90 | | 7.4 | | 5.3 | |
| | 0.5 Gm./lb. | 1.010 | 3.70 | 0.55 | 6.4 | 5.0 | 1.40 | 99.00 | 4.4 | 76 | 1.2 | 51.0 | 9.3 | 6.3 | 1.7 |
| 633 | KCl, | 1.032 | 0.44 | 1.30 | | 36.0 | | 1.10 | | 260 | | 11.0 | | 10.0 | |
| | 0.5 Gm./lb. | 1.019 | 1.20 | 1.20 | 2.5 | 18.0 | 1.40 | 80.00 | 270.0 | 205 | 2.1 | 42.0 | 10.0 | 8.4 | 2.2 |
| 658 | KCl, | 1.031 | 0.80 | 1.40 | | 26.0 | | 122.00 | | 208 | | 16.0 | | 6.3 | |
| | 0.5 Gm./lb. | 1.020 | 2.00 | 2.00 | 3.6 | 12.0 | 1.10 | 179.00 | 3.7 | 155 | 1.9 | 134.0 | 21.0 | 6.3 | 2.5 |
| 756 | KCl, | 1.027 | 0.76 | 0.31 | | 22.0 | | 131.00 | | 254 | | 85.0 | | ... | |
| | 0.5 Gm./lb | 1.014 | 2.40 | 0.26 | 2.6 | 7.7 | 1.10 | 154.00 | 3.7 | 108 | 1.3 | 101.0 | 3.8 | ... | .. |
| 759 | KCl, | 1.035 | 0.54 | 0.39 | | 35.0 | | 2.00 | | 411 | | 37.0 | | ... | |
| | 0.5 Gm./lb. | 1.027 | 1.00 | 0.99 | 4.7 | 20.0 | 1.10 | 72.00 | 67.0 | 296 | 1.3 | 103.0 | 5.2 | ... | .. |
| 781 | KCl, | 1.020 | 0.64 | 5.00 | | 48.0 | | 0.64 | | 186 | | 16.0 | | 6.3 | |
| | 0.65 Gm./lb. | 1.012 | 5.50 | 1.60 | 2.8 | 5.4 | 0.97 | 92.00 | 1,230.0 | 148 | 6.6 | 142.0 | 78.0 | 6.3 | 8.6 |
| 633 | KCl, 0.5 Gm./ lb. + biotin | 1.030 | 0.56 | 2.10 | | 25.0 | | 48.00 | | 278 | | 14.0 | | 11.0 | |
| | | 1.019 | 1.60 | 1.40 | 1.9 | 11.0 | 1.30 | 146.00 | 8.8 | 168 | 1.7 | 118.0 | 24.0 | 8.0 | 2.1 |
| 658 | KCl, 0.5 Gm./ lb. + biotin | 1.013 | 1.70 | 2.40 | | 15.0 | | 1.40 | | 135 | | 19.0 | | 8.4 | |
| | | 1.010 | 2.20 | 2.80 | 1.5 | 12.0 | 1.00 | 45.00 | 43.0 | 130 | 1.3 | 64.0 | 4.3 | 6.1 | 0.9 |
| 756 | NaCl, | 1.036 | 0.35 | 0.16 | | 30.0 | | 0.24 | | 354 | | 8.0 | | ... | |
| | 0.5 Gm./lb. | 1.033 | 0.46 | 0.26 | 2.1 | 30.0 | 1.30 | 2.60 | 14.0 | 397 | 1.4 | 22.0 | 3.5 | ... | .. |
| 759*** | NaCl, | 1.012 | 1.20 | 3.60 | | 11.0 | | 0.63 | | 134 | | 94.0 | | ... | |
| | 0.5 Gm./lb. | 1.009 | 2.50 | 2.30 | 1.3 | 9.4 | 1.80 | 22.00 | 72.0 | 98 | 1.5 | 80.0 | 1.8 | ... | .. |

*Upper line, preadministration; lower line, postadministration. **$C_2V_2/C_1V_1 = \Delta E$, change in absolute rate of excretion. ***Some accidental loss of urine.

TABLE 2—Change in Ratios of Absolute Renal Excretion of Several Elements with KCl, KCl-Biotin, and NaCl Administration

| Animal (No.) | Exper. | Ca : Mg* | Ca : Na | Ca : K | Ca : Cl | Ca : P | Mg : Na | Mg : K | Mg : Cl | Mg : P | K : Na | K : Cl | K : P | Na : Cl | Na : P | Cl : P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 781 | KCl, 0.5 Gm./lb. | 0.024 | 0.0040 | 0.00130 | 0.0160 | 0.023 | 0.160 | 0.058 | 0.700 | 1.00 | 2.80 | 12.0 | 17 | 4.3000 | 6.10 | 1.4 |
| | | 0.110 | 0.0060 | 0.00710 | 0.0110 | 0.087 | 0.052 | 0.068 | 0.110 | 0.83 | 0.77 | 1.5 | 12 | 1.9000 | 16.00 | 8.0 |
| 633 | KCl, 0.5 Gm./lb. | 0.036 | 1.2900 | 0.00500 | 0.1200 | 0.130 | 33.000 | 0.140 | 3.300 | 3.60 | 240.00 | 24.0 | 26 | 0.1000 | 0.11 | 1.1 |
| | | 0.064 | 0.0150 | 0.00570 | 0.0270 | 0.140 | 0.230 | 0.090 | 0.430 | 2.20 | 2.60 | 4.8 | 25 | 1.9000 | 9.60 | 5.1 |
| 658 | KCl, 0.5 Gm./lb. | 0.052 | 0.0110 | 0.00660 | 0.0900 | 0.220 | 0.210 | 0.130 | 1.600 | 4.20 | 1.70 | 13.0 | 33 | 7.5000 | 20.00 | 2.6 |
| | | 0.170 | 0.0110 | 0.01300 | 0.0150 | 0.310 | 0.067 | 0.078 | 0.100 | 1.70 | 0.87 | 1.2 | 24 | 1.3000 | 28.00 | 21.0 |
| 756 | KCl, 0.5 Gm./lb. | 0.014 | 0.0020 | 0.00120 | 0.0037 | .... | 0.168 | 0.087 | 0.260 | ... | 1.90 | 3.0 | .. | 1.5000 | .... | ... |
| | | 0.035 | 0.0017 | 0.00250 | 0.0026 | .... | 0.048 | 0.071 | 0.077 | ... | 0.68 | 1.0 | .. | 1.5000 | .... | ... |
| 759 | KCl, 0.5 Gm./lb. | 0.011 | 0.1900 | 0.00095 | 0.0110 | .... | 17.500 | 0.085 | 0.950 | ... | 210.00 | 11.0 | .. | 0.0540 | .... | ... |
| | | 0.056 | 0.0140 | 0.00330 | 0.0100 | .... | 0.280 | 0.068 | 0.190 | ... | 4.10 | 2.9 | .. | 0.7000 | .... | ... |
| 781 | KCl, 0.65 Gm./lb. | 0.010 | 7.8000 | 0.02700 | 0.3200 | 0.300 | 76.000 | 0.260 | 3.100 | 7.80 | 290.00 | 12.0 | 30 | 0.0400 | 0.10 | 2.5 |
| | | 0.290 | 0.0170 | 0.01100 | 0.0110 | 0.250 | 0.074 | 0.037 | 0.038 | 0.83 | 1.60 | 1.0 | 23 | 0.6500 | 14.00 | 22.0 |
| 633 | KCl, 0.5 Gm./lb. + biotin | 0.086 | 0.0450 | 0.00770 | 0.1500 | 0.190 | 0.520 | 0.090 | 1.800 | 2.30 | 5.80 | 20.0 | 25 | 3.5000 | 4.40 | 1.3 |
| | | 0.120 | 0.0090 | 0.00820 | 0.0120 | 0.170 | 0.077 | 0.067 | 0.100 | 1.40 | 1.20 | 1.4 | 21 | 1.2000 | 18.00 | 15.6 |
| 658 | KCl, 0.5 Gm./lb. + biotin | 0.160 | 1.7000 | 0.01800 | 0.1300 | 0.290 | 11.000 | 0.110 | 0.810 | 1.90 | 96.00 | 7.2 | 17 | 0.0750 | 0.17 | 2.3 |
| | | 0.240 | 0.0630 | 0.02200 | 0.0440 | 0.480 | 0.260 | 0.091 | 0.180 | 2.00 | 2.90 | 2.0 | 22 | 0.7000 | 0.76 | 11.0 |
| 756 | NaCl, 0.5 Gm./lb. | 0.005 | 0.6700 | 0.00450 | 0.0020 | .... | 125.000 | 0.085 | 0.380 | ... | 1490.00 | 4.5 | .. | 0.0030 | .... | ... |
| | | 0.009 | 0.1900 | 0.00070 | 0.0120 | .... | 12.000 | 0.076 | 1.400 | ... | 152.00 | 18.0 | .. | 0.1200 | .... | ... |
| 759** | NaCl, 0.5 Gm./lb. | 0.230 | 5.7000 | 0.02700 | 0.0380 | .... | 17.000 | 0.082 | 0.120 | ... | 212.00 | 1.4 | .. | 0.0067 | .... | ... |
| | | 0.250 | 0.1100 | 0.02400 | 0.0290 | .... | 0.440 | 0.100 | 0.120 | ... | 4.40 | 1.2 | .. | 0.2600 | .... | ... |

*Upper line, preadministration; lower line, postadministration. **Some accidental loss of urine.

nitude in either the water or the saline diuresis used.

Electrocardiographic observations* during four of the potassium and potassium-biotin trials showed only slight changes in three of the four trials and appeared to indicate that the blood levels of potassium attained were not markedly toxic to the heart. Toxic effects on the heart which could affect kidney function were thus apparently minimal. No electrocardiographic changes were noted when similar doses of sodium were given to 2 animals.

## DISCUSSION

Under the conditions used, it appeared that rise in plasma potassium occurred promptly after oral administration and that a major accompaniment of this change was elevation of plasma sodium. The latter presumably explained the increase in absolute renal excretion of sodium which appeared marked in some animals following potassium administration. Miller[15,16,17] found that administration of potassium salts increased the excretion of sodium for a time in swine and in rats. In man, sodium is lost in potassium chloride diuresis and vice versa.[27] Winkler and Smith[32] found that in dogs potassium injection caused a marked increase in sodium excretion. Baldwin *et al.*[4] suggest that in dogs a mutually inhibitory effect of potassium and sodium ions on tubular reabsorption is present.

In the present study with cattle, the reciprocal change, *i.e.*, potassium excretion when sodium was given, was not marked. This would tend to indicate that the bovine kidney may distinguish efficiently between these two cations.

Peters[19] noted that work to the date of his review indicated that administration of potassium causes a transitory sodium diuresis, perhaps due to the potassium being taken up by cells, leaving its associated ion to combine with sodium in extracellular fluids. Darrow and Pratt in a recent review[6] noted that there was usually a reciprocal relationship between intracellular sodium and potassium.

An interesting observation was the apparent constancy of absolute urinary magnesium excretion in the cow under the conditions used. It was noted previously that

*The electrocardiographic observations were made as a part of a continuing study on cardiac function, and the authors will include more complete records along with records from other animals in a separate publication at a future time.

in water diuresis the urine-magnesium concentration was inversely proportional to the volume of the urine;[21] thus, the absolute excretion remained the same. A similar relationship appeared to hold in the potassium, potassium-biotin, and sodium diureses. Control by the bovine kidney of the plasma level of this important regulatory ion seemed, in general, quite efficient under the experimental conditions used. A ready explanation would thus not be afforded for the hypomagnesemia reported by various investigators in cases of tetany in cattle soon after feeding on young and quick-growing grass and wheat high in potassium and water.[1-3, 7, 8, 10-14, 18, 20, 23, 24, 29, 30]

The absolute excretion of calcium, and to some extent of phosphorus, appeared to be increased by the saline diureses imposed, but to a degree not differing greatly from the increased absolute excretion of these elements with water diuresis. Curme *et al.*[5] noted that in sheep high potassium intake was associated with decreased calcium retention. No explanation is at hand for the regular rise in serum inorganic phosphate seen upon potassium or sodium administration.

No marked differences were apparent in repetitions of potassium trials in 2 animals where the potassium was preceded by large doses of biotin.

## SUMMARY

1) Similar doses of potassium chloride given alone and preceded by biotin and high sodium chloride intake were compared in lactating dairy cattle.

2) Plasma sodium showed a consistent rise with potassium chloride administration. A concomitant rise in absolute renal excretion of sodium appeared marked in some animals.

3) Reciprocal changes in plasma and urine potassium when sodium was given were not marked.

4) The urine-magnesium concentration was inversely proportional to the volume of urine in the potassium, potassium-biotin, and sodium trials performed.

5) A regular rise in serum inorganic phosphate followed administration of potassium or sodium chloride.

6) Absolute renal excretion of calcium, and to some extent of phosphorus, appeared increased by the saline diureses imposed, but to a degree not differing greatly from the increased absolute excretion of these elements with water diuresis.

7) No marked differences were apparent in repetitions of potassium trials in 2 animals where potassium administration was preceded by large doses of biotin.

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# A Comparative Study of the Intradermal Johnin Test on Cattle Artificially and Naturally Sensitized with Mycobacterium Paratuberculosis

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JOHNE'S DISEASE in cattle is a chronic, insidious infectious disease caused by *Mycobacterium paratuberculosis*. This organism is an acid-fast staining bacterium, resembling the one causing tuberculosis in man and animals in many respects.

The disease is characterized clinically by periodic diarrhea, progressive emaciation and, pathologically, by thickening and formation of folds in the intestinal mucosa. The disease is especially prevalent in intensely cultivated agricultural districts where, in some herds, it causes considerable annual losses, especially in young cows.

## EXPERIMENTAL PROCEDURE

The yearlings used in this series of tests were 1 year old and weighed about 400 lb. They were in a good growing state of flesh and remained so throughout the experiment. Three of the group were sensitized by giving 60 cc. of a mineral oil suspension containing 1 Gm. of the viable organisms of *M. paratuberculosis* through a cannula inserted into the peritoneal cavity high in the right flank region. First tests were made thirty days after the animals were given the dose of sensitizing organisms. The hair was clipped with extra fine tooth clippers from four 5-in. areas at least 4 in. apart, running longitudinally on each side of the animals. Four such rows were made on each side of these 3 artificially sensitized animals.

Five naturally sensitized yearlings were selected from a herd of about 400 cattle that had a yearly mortality rate from Johne's disease of 1 to 5 per cent. This herd has been known to be infected with Johne's disease since 1927. Because of the great value of these purebred yearlings, there were only three areas clipped from each row across the sides, which explains the deviation from the pattern used in the artificially sensitized yearlings. A new or unused area on the sides of the neck was tested each time to act as a control.

Millimeter readings were made with the Kummer and Johnson[1] dermal thickness gauge, at the time of intradermal injections of johnin and forty-eight hours later. The johnin used in these tests was made by the Regional Animal Disease Research Laboratory at Auburn, Ala. Two-tenths cubic centimeters of johnin was injected into each clipped area. The top, or first row, nearest the vertebral column was injected at one-week intervals in the same site. The second row at two-week intervals, the third row at three-week intervals, and the fourth row at four-week intervals. Twenty-seven gauge, ⅜-in. needles were used for all injections. An increase in skin thickness of 3 mm. or more was classed as positive and represented by a plus (+) sign. An increase from 2 to 3 mm. was classed as suspicious and represented by a plus and minus (±) sign. All increases less than 2 mm. were classed as negative and represented by a minus (—) sign.

## EXPERIMENTAL RESULTS

Table 1 gives the results of tests at one-week intervals on the artificially sensitized, naturally sensitized, and control animals. It also gives the number of positive, suspicious, and negative tests and their relative percentages on continually used and unused or control areas.

Table 2 gives the results of tests at two-week intervals on the artificially sensitized, naturally sensitized, and control animals. It also gives the number of positive, suspicious, and negative tests and their relative percentages on continually used and unused or control areas.

Table 3 gives the results of tests at three-week intervals on the artificially sensitized,

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This is a coöperative project between the Department of Veterinary Science, Louisiana State University, Baton Rouge, and U. S. Department of Agriculture, Bureau of Animal Industry, Regional Animal Disease Research Laboratory, Auburn, Ala.

Dr. Sikes was formerly at Louisiana State University, Baton Rouge, where the experimental work was done on naturally sensitized cattle. At present, he is at the Department of Animal Husbandry and Veterinary Science, University of Tennessee, Knoxville. Dr. Johnson was formerly in charge of Johne's disease research, U. S. Department of Agriculture, Bureau of Animal Industry, Regional Animal Disease Research Laboratory, Auburn, Ala., and is now in charge of the Bureau of Animal Industry, Beltsville Station, Beltsville, Md. Dr. Oglesby is head of the Department of Veterinary Science, Louisiana State University.

TABLE 1—Results of Tests at One-Week Intervals

| | No. animals | No. testing areas | No. tests each area | Total tests completed | Positive | | Suspicious | | Negative | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | No. tests | % | No. tests | % | No. tests | % |
| Artificially sensitized | 3 | 30 | 10 | 300 | 169 | 56.33 | 49 | 16.34 | 82 | 27.33 |
| Unused areas on artificially sensitized | 3 | 30 | 1 | 30 | 30 | 100.00 | 0 | 0.00 | 0 | 0.00 |
| Naturally sensitized | 5 | 30 | 10 | 300 | 17 | 5.66 | 13 | 4.34 | 270 | 90.00 |
| Unused areas on naturally sensitized | 5 | 50 | 1 | 50 | 25 | 50.00 | 25 | 50.00 | 0 | 0.00 |
| Control animal | 1 | 6 | 10 | 60 | 0 | 0.00 | 0 | 0.00 | 60 | 100.00 |
| Unused areas on control animal | 1 | 10 | 1 | 10 | 0 | 0.00 | 0 | 0.00 | 10 | 100.00 |

TABLE 2—Results of Tests at Two-Week Intervals

| | No. animals | No. testing areas | No. tests each area | Total tests completed | Positive | | Suspicious | | Negative | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | No. tests | % | No. tests | % | No. tests | % |
| Artificially sensitized | 3 | 30 | 9 | 270 | 203 | 75.18 | 30 | 11.11 | 37 | 13.71 |
| Unused areas on artificially sensitized | 3 | 27 | 1 | 27 | 26 | 96.30 | 0 | 0.00 | 1 | 3.70 |
| Naturally sensitized | 5 | 30 | 9 | 270 | 24 | 8.88 | 6 | 2.22 | 240 | 89.00 |
| Unused areas on naturally sensitized | 5 | 45 | 1 | 45 | 34 | 75.55 | 11 | 23.45 | 0 | 0.00 |
| Control animal | 1 | 6 | 9 | 54 | 0 | 0.00 | 0 | 0.00 | 54 | 100.00 |
| Unused areas on control animal | 1 | 9 | 1 | 9 | 0 | 0.00 | 0 | 0.00 | 9 | 100.00 |

TABLE 3—Results of Tests at Three-Week Intervals

| | No. animals | No. testing areas | No. tests each area | Total tests completed | Positive | | Suspicious | | Negative | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | No. tests | % | No. tests | % | No. tests | % |
| Artificially sensitized | 3 | 30 | 6 | 180 | 148 | 82.22 | 18 | 10.00 | 14 | 7.78 |
| Unused areas on artificially sensitized | 3 | 18 | 1 | 18 | 18 | 100.00 | 0 | 0.00 | 0 | 0.00 |
| Naturally sensitized | 5 | 30 | 6 | 180 | 25 | 13.88 | 18 | 10.00 | 137 | 76.12 |
| Unused areas on naturally sensitized | 5 | 30 | 1 | 30 | 22 | 73.33 | 8 | 26.67 | 0 | 0.00 |
| Control animal | 1 | 6 | 6 | 36 | 0 | 0.00 | 0 | 0.00 | 36 | 100.00 |
| Unused areas on control animal | 1 | 6 | 1 | 6 | 0 | 0.00 | 0 | 0.00 | 6 | 100.00 |

TABLE 4—Results of Tests at Four-Week Intervals

| | No. animals | No. testing areas | No. tests each area | Total tests completed | Positive | | Suspicious | | Negative | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | No. tests | % | No. tests | % | No. tests | % |
| Artificially sensitized | 3 | 30 | 5 | 150 | 133 | 88.67 | 11 | 7.33 | 6 | 4.00 |
| Unused areas on artificially sensitized | 3 | 15 | 1 | 15 | 15 | 100.00 | 0 | 0.00 | 0 | 0.00 |
| Naturally sensitized | 5 | 30 | 5 | 150 | 25 | 16.66 | 21 | 14.00 | 104 | 69.34 |
| Unused areas on naturally sensitized | 5 | 25 | 1 | 25 | 21 | 84.00 | 4 | 16.00 | 0 | 0.00 |
| Control animal | 1 | 6 | 5 | 30 | 0 | 0.00 | 0 | 0.00 | 30 | 100.00 |
| Unused areas on control animal | 1 | 5 | 1 | 5 | 0 | 0.00 | 0 | 0.00 | 5 | 100.00 |

naturally sensitized, and control animals. It also gives the number of positive, suspicious, and negative tests and their relative percentages on continually used and unused or control areas.

Table 4 gives the results of tests at four-week intervals on the artificially sensitized, naturally sensitized, and control animals. It also gives the number of positive, suspicious, and negative tests and their relative percentages of continually used and unused or control areas.

## DISCUSSION

It is a common observation in testing with intradermal johnin herds of cattle that are known to be infected with Johne's disease that they do not react on repeated tests with a uniform regularity or necessarily show the same degree of intensity in size of reactions.

An attempt was made to try to find an answer to the following questions.

1) How does the sensitivity of naturally and artificially sensitized cattle compare when repeated tests are made in the same site?

2) Does a partial local desensitization take place in cattle affected with Johne's disease when repeated intradermal johnin tests are made in the same site?

3) Are cattle that are affected with Johne's disease sensitive to the intradermal test, even though they fail to react on repeated tests in the same site?

4) In testing a herd for Johne's disease, how much time should be allowed between tests if they are made in the same site?

Rider[2] found that, in 1,100 double intradermal tuberculin tests on the side of the neck of naturally sensitized tuberculous cattle, 90 per cent of the negative reactions had a swelling not over 2 mm. at forty-eight hours and the total increase was not over 3 mm. In 90 per cent of the positive reactions, the increase was more than 3 mm. at forty-eight hours and the total increase was more than 5 mm.

From tables 1, 2, 3, and 4, it is noted that there is a greater number of positive and suspicious reactions in the artificially sensitized animals than in the naturally sensitized cattle. Their relative percentages are: 1) Artificially sensitized, retested at one-week intervals: 56.33 per cent of all tests positive and 16.34 per cent suspicious while, in the naturally sensitized, there were only 5.66 per cent of all tests positive and 4.34 per cent suspicious. (2) Artificially sensitized, retested at two-week intervals: 75.18 per cent of all tests positive and 11.11 per cent suspicious while, in the naturally sensitized, there were only 8.88 per cent of all tests positive and 2.22 per cent suspicious. (3) Artificially sensitized, retested at three-week intervals: 82.22 per cent of all tests positive and 10.00 per cent suspicious while, in the naturally sensitized, there were only 13.88 per cent of all tests positive and 10.00 per cent suspicious. (4) Artificially sensitized, retested at four-week intervals: 88.67 per cent of all tests positive and 7.33 per cent suspicious while, in the naturally sensitized, there were only 16.66 per cent positive and 14.00 per cent suspicious. These data suggest that these artificially sensitized animals did maintain a much higher degree of sensitivity than the naturally sensitized.

Wilson, Schwabacher, and Maier[3] were able to desensitize partially guinea pigs which were sensitized with heat-killed tubercle bacilli by daily injections of old tuberculin. They believe that desensitization probably is never complete in such animals. Apparently, it acts not by abolishing allergy but by converting a high into a low degree of allergy. From tables 1, 2, 3, and 4, it is interesting to note that the number of positive tests were markedly reduced in the naturally sensitized animals after the initial injections were made upon the sites. For example, from table 1, we find only 17 positive out of 300 tests; all these occurred on the first or initial test and none occurred thereafter. In table 2, we find a similar picture with 24 positive out of 270 tests. All of these positive tests occurred on the first test and none thereafter. A little different picture occurs in table 3, in which there are 25 positive out of 150 completed tests. All of these positive tests occurred on the first two tests. In table 4, a somewhat identical picture occurs with 25 positive out of 150 completed tests. All these positive tests occurred on the first two tests and none thereafter. It is obvious that the degree of sensitivity was greater after a lapse of three or four weeks between tests than after a lapse of one week. The number of positive tests was 17 when retested at one-week intervals and 25 when retested at four-week intervals. Even though there is an increase in

sensitivity, it is evident that complete sensitivity had not been regained.

The relative percentages of the artificially sensitized animals, as shown in tables 1, 2, 3, and 4, are: At one-week interval, 56.33 per cent; at two-week intervals, 75.18 per cent; at three-week intervals, 82.22 per cent; and at four-week intervals, 88.67 per cent. It would seem then that the artificially sensitized animal, with a relatively large available supply of antigenic substances, does regain sensitivity in the sites where repeated intradermal johnin injections are made more quickly than naturally sensitized animals.

It is noted that the animals gave positive or suspicious reactions throughout the experiment in the control or unused areas. Of great interest is the lag phase or reduced sensitivity in these areas of the naturally sensitized animals. This occurred immediately after the first injections were made in all four rows on each side of the animals. This reduced sensitivity lasted for one month after the initial injections were made. A possible explanation lies in the fact that a total of 4.8 cc. of johnin was used in the initial injections on each animal and a partial general desensitization resulted because of this large amount of johnin.


## SUMMARY

1) It is shown that the sensitivity of cattle artificially sensitized with *Mycobacterium paratuberculosis* is greater than naturally sensitized ones when repeated intradermal injections are made in the same site at one-, two-, three-, and four-week intervals.

2) These data suggest that a partial local desensitization does take place when repeated injections of intradermal johnin are made in the same site.

3) From these data, it seems that cattle affected with Johne's disease will react to intradermal johnin in a previously unused site, even though they fail to react when repeated tests are made in the same site.

4) These data suggest that a lapse of time longer than four weeks is necessary before retesting a herd of cattle for Johne's disease, if the same testing site is to be used each time.


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# An Attempt To Immunize Sheep During an Outbreak of Listeriosis

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ATTEMPTS AT immunization against listeriosis have been reported by various investigators. Graham, Levine, and Morrill[1] have summarized the studies of immunization against experimental infection in laboratory animals and the use of so-called bacterins (suspensions of *Listeria monocytogenes* killed by various methods) in outbreaks of listeriosis in sheep on farms. In general, the various procedures have failed to immunize animals against either experimental or natural exposure to the infection. Olson, Cook, and Blore[2] found that sheep exposed to live culture of *L. monocytogenes* two or more times were able to resist the intracarotid inoculation of the live organism. A single exposure of sheep to a living culture of *L. monocytogenes* appeared to prevent fatal infection in some but not all animals given the organism by way of the carotid artery. The injection of live *L. monocytogenes* into the cerebral hemisphere of sheep which had previously had several exposures to the organism resulted in death of most of the sheep.

The pathogenesis of listeriosis is not understood. The origin and mode of spread of the infection in field outbreaks is likewise obscure. The Nebraska Agricultural Experiment Station, for several years, has conducted certain feeding trials with sheep at its substation at Scottsbluff, Neb. Usually, approximately 500 sheep have been purchased as feeders in November, fed until the following March, and then sold for slaughter. The feedlots have been used for a number of years. Outbreaks and losses from listeriosis have occurred during each feeding period for at least the past four years. The extent of mortality has varied from year to year.

In anticipation of another outbreak, a plan was developed to ascertain the effect of immunization with live cultures of *L. monocytogenes*. Since previous work had demonstrated that two such injections would immunize sheep against intracarotid exposure to the organism, it seemed that this procedure done in the early stages of a field outbreak might produce results which would shed some light on the pathogenesis of a natural outbreak of listeriosis. These observations constitute the basis for this report.

## MATERIALS AND METHODS

In order to test the ability of *L. monocytogenes*, strain 136, to immunize against intracarotid exposure, subsequent to the work referred to previously, further trials were done during the fall of 1949. Two sheep were given heat-killed cultures (exposed to 65 C. for four and one-half hours); 2 sheep received 1 per cent formolized culture (1% formalin-killed *L. monocytogenes* in six hours); and 2 sheep were given live cultures. These preparations were given in 5.0-ml. amounts, subcutaneously. Two injections were made seven days apart. Seven days after the last subcutaneous injection, each sheep received a 24-hour tryptose broth culture (0.05 ml. per lb. of body weight) via the carotid artery. One sheep which received heat-killed culture and 1 sheep which received formalin-killed culture died in seven and sixteen days, respectively after intracarotid exposure. The remaining 4 sheep survived. These results indicate that strain 136 in the live state still possessed the ability to immunize against intracarotid exposure.

On Nov. 4, 1949, 540 yearling sheep were delivered to the substation from New Mexico. The sheep were put on pasture for two weeks and on November 18 were divided into 12 groups of 45 sheep each. The lots were arranged in two parallel rows, six lots being on each side of a center driveway. Each lot was provided with its own feed and hay racks and water tank. Each group of sheep received a different ration. The disease occurred in some

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sheep of each of the lots, but no correlation could be made between the ration and the disease. The first evidence of listeriosis was observed December 13, when 1 sheep was noted dull and listless. It died two days later. The body temperature prior to death was 107 F. The last case of the outbreak died sixty-five days later. Thus, the outbreak lasted for slightly more than two months. The nature of the disease was established by isolation of *L. monocytogenes* from fatal cases.

The culture vaccine was prepared by growing *L. monocytogenes*, strain 136, in tryptose broth medium. Strain 136 was isolated from a sheep that died with listeriosis during the 1947 outbreak of the disease at the Scottsbluff substation. The cultures were incubated for twenty-four hours at 37 C., then placed in rubber-stoppered serum bottles and used for immunization about twenty-four hours later. Those sheep selected for immunization received two 2.5-ml. doses of the culture vaccine, given subcutaneously one week apart. The first dose was administered December 21 and the second December 28.

At the time of the first administration of culture vaccine, the sheep were divided into two groups, one of which was left untreated as a control group. Previous experience had indicated that sheep with listeriosis are likely to have an elevated body temperature. Rectal temperatures were measured in all of the sheep the day before the first vaccination. An arbitrary value of 103 F. was used as the dividing point. Four groups of sheep in each of the twelve lots were selected. One group contained those sheep with a body temperature of less than 103 F., a second group those with 103 F. or higher body temperature. These first two groups received the culture vaccine. The third group of sheep had a body temperature of less than 103 F., and the fourth group had a body temperature of 103 F. or higher. Groups 3 and 4 did not receive the culture vaccine. In each of the twelve lots, groups 1 and 3 were of the same approximate number, and groups 2 and 4 were of the same approximate number. A total of 271 sheep were vaccinated, and 268 sheep represented the unvaccinated controls.

Rectal temperatures were taken on all of the sheep seven times (on Dec. 20, 23, 27, 30, Jan. 6, 13, and 20). In order to have somewhat comparable data on the rectal temperatures, 100 sheep of the flock at Lincoln were used for study. Beginning on December 23 and continuing to January 10, temperatures were taken six times at intervals of two to seven days.

Bacteriologic examinations were made of the brains of most and cultures were attempted from the spleens and livers of some of the sheep that died. The technique of examination was as follows. Portions (0.25 cc. to 1 cc.) of the cerebellum, cerebrum, and brain stem were placed in different tubes of tryptose broth medium and ground. At the same time, portions from the same parts of the brain were put in a 50 per cent glycerin and salt solution for preservation in the refrigerator. If, at the end of three weeks, *L. monocytogenes* had not been isolated from the primary attempts at culture, then the material preserved in 50 per cent glycerin was ground in tryptose broth medium for a second attempt at isolation of the organism.[3]

During the course of the outbreak, some observations were made on the reaction of leucocytes in the blood, therapeutic attempts with penicillin, and therapeutic attempts with the serum and blood from sheep recovered from the disease.

## RESULTS

Listeriosis was recognized on the basis of physical symptoms in 49 of the sheep, or 11 per cent of the flock (table 1). Ten sheep had died before half of the flock received the culture vaccine. These 10 sheep have been counted with the nonvaccinated half of the flock. In the week following first vaccination, only 1 sheep developed listeriosis among the 271 sheep receiving the culture vaccine. In the same interval, 5 of the 268 control sheep developed the dis-

Fig. 1—Sheep recovered from listeriosis with permanent torticollis. Sheep 833 on the right and sheep 908 on the left. Photograph taken about six months after illness.

ease. Following the second administration of culture vaccine, 28 sheep receiving the culture vaccine became affected with listeriosis and 24 died. Of this number, 21 showed the first symptoms within ten days after receiving the second dose of culture vaccine. During the same interval, only 5 of the control sheep developed the disease and died.

The data were examined to study the duration or course of the disease. This was the interval between the time symptoms were first noted and death of the animal. Among the sheep not vaccinated and receiving no therapeutic treatment, death usually occurred in less than one or two days (3 died in less than one, 8 died in one day, 3 in two days, and 1 after 8 days). Among those sheep vaccinated and dying from listeriosis, the course of the disease was slightly prolonged (3 died in less than one day, 4 died in one day, 5 in two days, 6 in three days, 2 in four days, 1 in seven days, and 1 after thirty-seven days).

The symptoms of listeriosis in the early part of the outbreak and among sheep not vaccinated were, in general, typical of the usual field cases of the disease. Early symptoms were dullness, stiffness of the front legs, unsteady gait, poor vision, and elevation of body temperature. Frequently, there was a tenacious mucous discharge from the nostrils and paralysis of the lower lip with drooling. Often, one of the first evidences of disease was failure to eat. Later, the muscular incoördination was more marked, and often the sheep would lean against the fence for support. When forced to move, the sheep would tend to circle. In some individuals, the head and neck would be turned to one side. There was no relaxation of the torticollis after those sheep were unable to stand. A cloudiness of the cornea of one or both eyes was observed in some cases. The depression was progressive, leading to coma. As previously mentioned, the course of the disease was very rapid, lasting only a few hours in some cases. Diarrhea was noted in a few cases.

TABLE I—Morbidity, in Relation to Time, from Listeriosis During an Outbreak Among 540 Sheep, Beginning Dec. 13, 1949*

| Days of outbreak | Vaccinated (271 sheep) | Non-vaccinated (268 sheep) |
|---|---|---|
| 1 | .. | 1 |
| 3 | .. | 1 |
| 5 | .. | 2 |
| 7 | .. | 3 |
| 8 | .. | 1 |
| 9 | .. | 2 |
| First vaccination | .. | .. |
| 10 | .. | 1 |
| 11 | .. | 1+2** |
| 13 | 1 | 1 |
| Second vaccination | .. | .. |
| 16 | 3 | 1 |
| 17 | 1 | .. |
| 18 | 1 | .. |
| 21 | 1 | .. |
| 22 | 2 | .. |
| 23 | 2 | .. |
| 24 | 5+1** | .. |
| 25 | 4 | .. |
| 27 | 1 | .. |
| 28 | 1+1** | 1 |
| 29 | 1 | 1 |
| 32 | 2** | .. |
| 33 | .. | 1 |
| 38 | .. | 1 |
| 39 | 1 | .. |
| 65 | 1 | .. |
| Total | 29 | 20 |

*Diagnosis by physical symptoms, confirmed by isolation of organism from 39 sheep that died.
**5+1 indicates 5 died and 1 sick recovered.

One case was of particular interest for the animal was apparently normal, having come to feed a few hours prior to its death. While running with its pen mates as they were being watched, the sheep fell suddenly and died in a few minutes. Blood counts were taken immediately and the values for leucocytes were characteristic of listeriosis. The visceral organs were apparently normal, except for the heart muscle which was faintly streaked with gray areas. Subsequent bacteriologic examination failed to demonstrate *L. monocytogenes* in any part of the brain, but the organism was found in the heart muscle, the liver, and the spleen. Death, in this case, was probably due to heart failure because of localization and damage to the heart muscle by *L. monocytogenes*. Such localization in the heart muscle has been reported as fairly common in listeriosis of the chicken.[4]

The sheep developing the disease after vaccination displayed somewhat different symptoms, particularly in those cases that lived three or more days after first evidence of the disease. Mucous discharge from the nose was absent or scant. Some of these animals became unable to stand but their sensorium was clear and they continued to eat and drink when feed and water were brought to them. Their symptoms were more those of a transverse myelitis than an encephalitis, but no histologic studies were made to confirm or deny this conception. It was a peculiar sight to see such a sheep down, flat on its side, yet with a bright eye and willing to eat when its head was supported so that it could get at feed

or water. The unresponsiveness and "lost" attitude of a sick sheep is well known.

Three sheep were treated by an attending veterinarian during the early part of the outbreak. These sheep were given penicillin and lived for two (sheep 1173), four (sheep 1167), and eight (sheep 1069) days. Since most sheep comparable to these died in two days or less, penicillin may have prolonged their lives. Counts of leucocytes were made on these 3 sheep, two days after the administration of penicillin. At this time, 1 animal (sheep 1069) was unable to stand and was nearly comatose, with a body temperature of 101.5 F. Its neutrophils numbered 4,233, lymphocytes 892, and myelocytes 186 cells per cmm. of blood. Another of the group (sheep 1173) had a keratitis of the left eye and was down and comatose, with a temperature of 103.0 F. Its leucocyte count was neutrophils 5,877, lymphocytes 1,771, and myelocytes 362 per cmm. of blood. A third animal (sheep 1167) that received penicillin was depressed, had paralysis of the lower lip with drooling, tended to circle, and had a temperature of 103.5 F. Its leucocyte count was 6,369 neutrophils, 3,160 lymphocytes, and 321 myelocytes per cmm. of blood. The numbers of leucocytes were typical of those found in sheep with experimentally produced listeriosis.[2]

Counts of leucocytes were made on 2 other sick sheep on the first day of illness. One of these (sheep 937) was depressed, nearly comatose, and unable to stand. The body temperature was 106.3 F. A count of leucocytes revealed 5,938 neutrophils, 2,826 lymphocytes, and 570 myelocytes per cmm. of blood. Sheep 937 died the same day on which symptoms were first observed. The other animal (sheep 1034) was dull and weak, with a temperature of 103.1 F. Its leucocyte count was 3,408 neutrophils, 1,236 lymphocytes, and 144 myelocytes per cmm. of blood. It died the next day. As may be noted, both of these sheep showed a relatively high number of neutrophils, characteristic of experimental listeriosis in sheep.[2]

Two other attempts at therapy of affected sheep were made. One of these utilized serum from an animal (sheep 6) that had been repeatedly exposed in the laboratory to cultures of *L. monocytogenes*. The other therapeutic attempts were with whole blood from sheep 240. Sheep 240 showed no physical signs of disease, but an elevated temperature and increased number of neutrophils on December 21 suggested that the animal had listeriosis at that time. Subsequent temperatures and blood counts on January 14 were normal, indicating recovery. The therapeutic trials were done on 7 sheep which showed symptoms of listeriosis following vaccination. Four of the sheep were treated with serum and 3 were treated with whole blood.

*Protocols of 4 Sheep Given Serum from Sheep 6.*—Counts of leucocytes were made on the 4 sheep prior to the intravenous administration of 50 cc. of serum and again twenty-four hours later.

Sheep 992 was first noted sick January 11. Rectal temperature was elevated January 6. It did not exhibit marked stiffness and

TABLE 2—Temperatures and Counts of Leucocytes on Sheep with no Physical Symptoms During the Outbreak of Listeriosis

| Sheep (No.) | Temperatures (degrees F. above 100) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 12/20 | 12/23 | 12/27 | 12/30 | 1/6 | 1/13 | 1/15 | 1/20 |
| 119 | 4.2 | 4.2 | 4.4 | 4.6 | 3.0 | 3.0 | 2.0 | 3.8 |
| 240 | 5.0 | 3.8 | 4.5 | 2.4 | 3.2 | 1.0 | 2.4 | 3.2 |
| 290* | 4.6 | 4.6 | 2.0 | 2.9 | 1.4 | 2.0 | 2.3 | 3.0 |
| 1067 | 4.2 | 4.2 | 3.0 | 3.0 | 2.5 | 3.6 | .. | 3.0 |
| 1000 | 4.2 | 2.8 | 1.9 | 3.0 | 2.2 | 2.0 | .. | 2.3 |
| 1024 | 2.4 | 4.2 | 3.2 | 2.4 | 4.7 | 2.0 | .. | 4.0 |
| 349 | 5.0 | 4.9 | 3.2 | 4.9 | 4.9 | 4.3 | .. | 3.6 |

| Sheep (No.) | Counts of leucocytes (cells per cmm.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Total | Neutrophils | Lymphocytes | Myelocytes | Total | Neutrophils | Lymphocytes | Myelocytes |
| | 12/21 | | | | 1/14 | | | |
| 119 | 10,775 | 8,135 | 2,182 | 269 | 5,625 | 2,180 | 2,883 | 183 |
| 240 | 7,225 | 3,588 | 3,251 | 199 | 8,375 | 2,743 | 4,962 | 272 |
| 290* | 6,200 | 2,139 | 3,767 | 171 | 5,375 | 1,411 | 3,655 | 134 |
| 1067 | 12,050 | 4,338 | 6,507 | 181 | 6,800 | 986 | 5,304 | 289 |
| 1000 | 5,900 | 1,947 | 3,599 | 221 | ..... | .... | .... | ... |
| 1024 | ..... | .... | .... | ... | 6,475 | 1,554 | 4,597 | 162 |
| 349 | ..... | .... | .... | ... | 10,450 | 1,959 | 7,890 | 209 |

*Mild symptoms of inappetence and incoördination December 27. This animal was included among those with listeriosis in tables 1 and 2.

was able to get up and eat. There was a corneal abscess in the left eye. The number of neutrophils on January 14 was indicative of listeriosis although rectal temperature was 103.3 F. The animal did not improve following administration of serum January 14 and died January 19.

**TABLE 3—Body Temperatures of Sheep in Outbreak of Listeriosis at Time of Administration of Culture Vaccine**

| Date | 271 sheep vaccinated 12/21 and 12/28 | 259 sheep not vaccinated |
|---|---|---|
| 12/20 | 103.0±0.7* | 103.0±0.8 |
| 12/23 | 104.1±0.3 | 103.4±0.9 |
| 12/27 | 102.9±0.9 | 102.8±0.7 |
| 12/30 | 102.9±0.8 | 102.9±0.7 |

*Mean and standard deviation.

Sheep 944 was stiff on January 4. Symptoms of listeriosis were progressive until it became unable to stand by January 14. The number of neutrophils was high (5,751 per cmm.) at that time, although body temperature was normal. Serum was given January 14. On January 15, there was no improvement of symptoms and the number of neutrophils remained elevated (6,610 per cmm.). The condition of this animal was worse January 19. It became comatose and died January 21.

Sheep 108 was first sick on January 6. Three to four days later it was able to stand. There was an occasional convulsive kicking of all four legs with only moderate coördination. There was slight opisthotonos. The right cornea was cloudy. Serum was given January 14. The number of neutrophils was high both before (4,413 per cmm.) and twenty-four hours (3,742 per cmm.) after serum administration. The sheep continued to eat although it was down. Its condition became worse and it died January 22.

Sheep 908 showed first symptoms January 14, on the day serum was given. There was a stiffness of all four legs. The number of neutrophils was high before (6,408 per cmm.) and twenty-four hours after (7,610 per cmm.) administration of serum. The condition of the sheep seemed slightly worse twenty-four hours after the serum was given. Movements of the legs were incoördinated when the sheep was struggling while being held. On January 20, the sheep was walking about as though in search of food. Prior to this, feed had been brought to the sheep. By February 2, the animal was considered recovered although there was a residual torticollis (fig. 1).

The only apparent effect of serum in these trials was a probable prolongation of life by a few days. Recovery of only 1 of 4 sheep thus treated is of questionable significance.

*Protocols of 3 Sheep Receiving Whole Blood from Sheep 240.* — About 30 cc. of whole citrated blood were given intravenously. Counts of leucocytes and temperatures were taken before and from 1 animal six hours after administration of the blood.

Sheep 1198 was first ill January 6 when the symptoms were mild. These were evident until January 11, when the animal had to be helped to its feet because of stiffness of the front legs. The appetite remained good. On January 14, stiffness of the front legs was not quite so marked. Body temperature was normal (101.5 F.) although the number of neutrophils (4,257 per cmm.) indicated active listeriosis infection. The number of neutrophils was reduced (2,704 per cmm.) six hours after administration of blood. Symptoms of listeriosis gradually disappeared, and the animal was apparently well on February 2.

Sheep 925 was first sick January 10. It was still able to stand January 14 although weak, and the head was turned to the left. The moderately elevated number of neutrophils (2,915 per cmm.) and body temperature (103.8 F.) were suggestive of listeriosis infection at this time. No improvement was apparent six hours after administration of blood but, by January 19, the animal was able to move about actively and search for food. It recovered completely without any residual symptoms.

Sheep 833 was first noted sick on January 14 when it had a stiffness of the legs. A high temperature (104.8 F.) and slightly elevated number (2,859 per cmm.) of neutrophils suggested listeriosis infection. The sheep was still eating. Six hours after the administration of blood, the body temperature was lowered one degree but there was no change in symptoms. The sheep became progressively worse during the next several days. It continued to eat and eventually recovered but had a permanent torticollis (fig. 1).

Control sheep 893 was first noted sick with stiffness of the legs on January 9. It was unable to stand a day or two later. On January 14, it was nearly comatose but

would eat a little. Blood counts at this time indicated an increased number (3,066 per cmm.) of neutrophils. The condition of the animal remained about the same until February 13 when it died.

Whole blood from a sheep suspected of having listeriosis and recovering without showing evident symptoms seemed to have a beneficial effect in 3 sheep with listeriosis. Two of these sheep recovered completely, and the other had a residual torticollis probably because of irreparable damage to nervous tissue.

*Suspected Listeriosis in Sheep That Did Not Show Physical Symptoms.*—Since temperatures were being taken of the entire group of sheep, opportunity existed to ascertain the possibility of mild, asymptomatic listeriosis. This was done by selecting a few animals, in which high temperatures were noted, and making counts of the leucocytes. The data on some sheep are given in table 2. Sheep 119, for example, had a body temperature of 104.2 F. or higher from December 20 to December 30. On December 21, the count of neutrophils was high and typical of that seen in listeriosis with definite symptoms. On January 14, the count of neutrophils was low and approached the normal level. No other evidence of illness was apparent but the data certainly suggest infection. Sheep 240 had an elevated body temperature from December 20 to December 27 and a moderately elevated number of neutrophils on December 21. Blood was taken from sheep 240 for use in the therapeutic trials previously mentioned. Sheep 290 was also suspected of having listeriosis on the basis of the body temperatures and the blood counts. In addition, it did show very mild symptoms of inappetence and incoördination for one day. Because of this, it was regarded as a definite case of listeriosis and has been included as such in other parts of this report. Sheep 1067 was also regarded as a case suspect of listeriosis. In this case, the decrease in neutrophils in twenty-four hours was more marked than in the others (table 2). Sheep 1000, 1024, and 349 were suspects of listeriosis on the basis of elevation of body temperature but blood counts were not conclusive in supporting this suspicion (table 2).

*Observations on Body Temperature.*—Analyses on the data of body temperatures was difficult because of the various factors involved.

The mean body temperature with standard deviation of 100 sheep at Lincoln, Neb., was 102.9 ± 0.7 F. This was based on six observations of each sheep made during a period of seventeen days. There was no significant variation of the temperature between the six days of observation. Another method of studying body temperature was to determine the spread or difference between the highest and lowest temperatures found in the individual sheep. This spread or difference was determined for each sheep, and the average for the entire group of sheep at Lincoln during the seventeen days was 1.4 ± 0.4 F.

TABLE 4—Isolation of Listeria Monocytogenes from Tissue of Sheep that Died

| | Brain stem | Cerebellum | Cerebrum | Liver | Spleen | Heart |
|---|---|---|---|---|---|---|
| Direct isolation only | 40/29* | 40/28 | 40/19 | 10/8 | 4/4 | 2/1 |
| From glycerinated tissue only | 21/10 | 15/3 | 26/3 | 3/1 | ... | ... |
| Both methods combined | 40/32 | 40/30 | 40/21 | 10/8 | 4/4 | 2/1 |

*40/29 = 40 attempts and *Listeria monocytogenes* isolated in 29.

In general, the sheep that died with listeriosis during the course of these observations did show an increase in body temperature. The increase tended to persist until the death of the animal. The body temperature of 17 of the 29 sheep that developed listeriosis following vaccination was elevated before the appearance of symptoms. In 2 of the sheep, the temperature was increased before the vaccination and perhaps they were sick at that time. Four of them had their initial elevation of body temperature following their first vaccination. Twelve sheep of the group displayed the first symptoms of listeriosis without previous increase of body temperature.

Examination of the data showed rather clearly that there was generally an increase of body temperature following the first dose of culture vaccine (table 3). The average increase as observed was rather small (one degree) and, in view of the standard deviation from the mean, was not statistically significant. The observations were made forty-eight hours following vaccination,

and higher temperatures may have been obtained at some earlier period, for example, at twenty-four hours. The temperature response did not occur following the second dose of culture vaccine.

Another way of estimating the temperature reaction following vaccination was to note the change of temperature on individual sheep between December 20 and December 23, that is, before the first culture vaccine was given to the sheep and two days after administration. The following results were obtained on the 271 sheep receiving the culture vaccine. In 23 per cent of the sheep, the temperature increase was two degrees or more; in 30 per cent, the increase was from 1.0 to 1.9 F.; in 23 per cent, the increase was from 0.3 to 0.9 F. In 15 per cent of the sheep, there was no change of temperature or a variation of only 0.2 F. In 6 per cent of the sheep, there was a decrease of temperature amounting to 0.3 to 0.9 F. and, in 3 per cent of the sheep, there was a decrease in temperature amounting to 1.0 to 1.9 F. There was, therefore, a rather definite increase of temperature in most of the sheep receiving the culture vaccine for the first time.

The spread of temperatures during the period from December 20 to January 20 was also determined for the sheep at Scottsbluff. Among the vaccinated sheep, this spread of temperatures amounted to $2.4 \pm 0.9$ F. In the group of sheep not vaccinated and yet involved in the outbreak, the spread of temperatures was $1.9 \pm 0.2$ F., or not greatly different than the spread noted among normal sheep at Lincoln.

*Attempts at Isolation of Listeria Monocytogenes.*—The results of attempts at isolation of the organism from sheep that died are presented in table 4. *Listeria monocytogenes* was isolated from 39 of the 40 sheep that died (actually 43 sheep died but material from only 40 was examined). It was found in one of the three parts of the brain routinely cultured in 38 of the cases. Direct isolation from fresh material was the best method. However, by use of glycerinated tissue (stored about three weeks in the refrigerator) some additional isolations were made. Although only four attempts were made to isolate the organism from the spleen, it was found in all of them. The number of observations is far too small to determine the significance or even frequency of the organism in the spleen in field cases of listeriosis.

## DISCUSSION

The attempt at immunization against listeriosis in the field outbreak must be regarded as unsuccessful. It would appear that the single dose of living *L. monocytogenes* organism had some beneficial effect in the first seven days following its administration. The second dose of culture vaccine seemed to have a disastrous effect in that morbidity from the disease was greatly increased. These results are difficult to understand. Previous experience[2] had demonstrated that the strain 136 of *L. monocytogenes* used was not pathogenic for normal sheep except when introduced directly into the brain or into the carotid artery. Other strains of *L. monocytogenes* have been reported as pathogenic when given intravenously[1] and subcutaneously in repeated doses.[3] Strain 136 has been used for multiple subcutaneous injection into normal sheep since it was used for the preparation of the culture vaccine. Such sheep did not die and, therefore, it may be assumed that the pathogenic power of the strain had not increased.

If listeriosis naturally develops as a result of *L. monocytogenes* gaining access to the brain via the carotid artery, different results should have been attained in the attempt to immunize during the outbreak. A possible explanation of the results in the vaccinated sheep might be that some additional etiologic factor existed and operated with the culture vaccine at the time of the second dose to cause the development of listeriosis. What the results might have been in a group of sheep receiving only one dose of culture vaccine must remain an open question. The suggested therapeutic benefit of whole blood from a sheep recovered from natural listeriosis was in marked contrast to the lack of effect of serum from sheep which had had repeated injections of a culture of *L. monocytogenes*. This should not be regarded as a therapeutic comparison of whole blood and serum. While the number of trials were small and the work must be repeated on a larger scale, it would appear that blood from the sheep with the natural disease contained something not present in the serum from the sheep experimentally exposed. This again suggests that something besides *L. monocytogenes* is involved in the pathogenesis of the natural disease. Graham, Levine, and Morrill[1] reported that antiserums against *L. monocytogenes* (prepared

in horses and cattle by injecting the organism) had no prophylactic value against subsequent intravenous exposure of rabbits, calves, or lambs. Julianelle[5] reported that antiserum from rabbits which had a high agglutination titer had no neutralizing effect on *L. monocytogenes* when the serum and culture were mixed prior to intraperitoneal injection into mice.

The clinical manifestations displayed during the outbreak were not greatly different from those reported by others.[6,3,1,7] The existence of asymptomatic listeriosis during the course of an outbreak is suggested by an increase of body temperature in some sheep, accompanied by an increase in the number of circulating neutrophils in the blood.

The development of residual symptoms, such as continuing torticollis in some of the sheep that recovered, must indicate irreparable damage to nervous tissue. Olafson[8] has noted evidence of permanent cerebral damage (similar to that in so-called "dummies" among horses surviving equine encephalomyelitis) in sheep recovered from natural and experimental infection. This type of damage might be due to some factor other than *L. monocytogenes*, since sheep can be given a large number of living organisms intravenously without any evidence of such nerve damage.[2]

Observations on leucocytes in the blood made during the course of the outbreak clearly indicate an increase in number of circulating neutrophils. In this respect, the natural disease is similar to experimental infection.[2] These observations confirm those of others.[6]

## SUMMARY

An attempt was made to immunize sheep during the course of an outbreak of listeriosis by giving approximately half of the flock of 540 sheep two subcutaneous injections of living *Listeria monocytogenes* cultures. Only 1 case of listeriosis occurred in the vaccinated sheep during one week following the first dose of culture vaccine. After the second dose of culture vaccine, more sheep developed the disease among the vaccinated group than among the nonvaccinated control group.

The existence of asymptomatic cases of listeriosis was suggested by some sheep with temporary elevations of body temperature and leucocytosis of neutrophils.

A sheep which had recovered from mild listeriosis in the early part of the outbreak provided whole blood which seemed to bring about recovery in 3 cases of listeriosis. Another sheep which had had a number of previous exposures to living *L. monocytogenes* provided serum which failed to halt fatal listeriosis in 3 of 4 sick sheep. Penicillin seemed to prolong life in 2 cases of natural listeriosis that finally had a fatal outcome.

Observations made during the course of the outbreak suggest the existence of some etiologic factor in addition to *L. monocytogenes* in the natural disease of sheep.

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# The Effect of Cortisone on Normal Sheep

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THE MANIFOLD effects of cortisone on human subjects and in small laboratory animals have been studied under a variety of conditions. The most pronounced metabolic effects have been: increased gluconeogenesis, protein catabolism, and deposition of hepatic glycogen;[15] increased absorption and storage of fat if cortisone were in excess;[22] a lowering of the renal threshold for glucose;[11] usually an increase in the excretion of potassium and chloride and a retention of sodium,[23] although sodium may be excreted or may be unaffected. In human male patients with Addison's disease, the 17-ketosteroid excretion has been observed to increase.[8] In the human female patient, cortisone may cause mild hirsutism.

The blood cell picture has been observed to change under the influence of administered cortisone. The changes reported in human patients and in experimental animals include increases in the total leukocytes[2] and in the percentage of circulating neutrophils and decreases in the percentages of lymphocytes and eosinophils.[16] As a result of prolonged administration of the hormone, the hemoglobin content of the blood may increase.

Because of the limited amount of cortisone available for investigational work, the experience with the hormone in large domestic animals has been limited. Doyle *et al.*[5] used cortisone in a therapeutic trial on swine affected with rheumatoid disease. Hatziolos and Shaw[9] reported that 1 case of bovine acetonemia treated with cortisone responded favorably. Further data on the effects of cortisone and other adrenal hormones on large animals are lacking.

The data of Cook *et al.*[4] and McCandless and Dye[13] indicated that there was a relative deficiency of the pituitary-adrenal mechanism for endogenous gluconeogenesis in ruminants. The biochemical and histopathological studies of Shaw[18, 19] showed that bovine acetonemia may be considered as a disease of adaptation, as elucidated by Selye.[17] Such information points to the necessity for more knowledge of the activities and effects of adrenal stereoids in large animals.

The present communication contains the results of a short-term study on the blood constituents and the electrocardiograms of sheep before and after the administration of cortisone.

## METHODS

Five nonpregnant 1-year-old ewes, weighing 90 to 110 lb., were used in this study. For several days prior to the experimental period, the animals were handled and bled, so that they became acclimated to the experimental procedure. Four ewes were subjected to electrocardiographic techniques. For a period of six days, the ewes were bled and electrocardiographic tracings were made at the same time each day to establish a normal range of values for the animals. Blood was taken by jugular venipuncture. Ammonium oxalate was used as an anticoagulant because both sodium and potassium were to be determined on the sample. Total blood cell counts and differential leukocyte counts were made. Hematocrits were determined. Blood analyses included the following: whole blood glucose by the method of Folin and Malmros[7] with the Shaffer-Hartmann-Somogyi zinc precipitant, and plasma chloride by the method of Whitehorn.[24] Plasma sodium and potassium were determined on the Beckmann flame spectrophotometer. Sodium was determined at 589 $\mu$ and potassium was determined at 767 $\mu$. Ringer's solution was used as a standardizing solution for the determination of these ions.

The electrocardiograms were made with a Sanborn viso-cardiette. Plate electrodes and standard limb leads were used throughout. The records were taken while the animal was standing in a specially constructed wooden chute designed to eliminate the pick up of interfering electrical currents.

Cortisone acetate* in saline suspension was injected intramuscularly into each of 4 of the animals according to the following schedule:

First and second days . . . 100 mg.
morning and evening.

From the School of Veterinary Medicine, University of California, Davis.

*Cortone acetate® was supplied to us through the courtesy of Merck & Co., Inc., Rahway, N. J.

Third, fourth, and fifth days . . . 100 mg. in the morning only.
Sixth and seventh days . . . 100 mg. morning and evening.

The fifth ewe received 200 mg. of cortisone on the morning of the first day only. In this animal, blood samples were drawn 3½, 5½, 12, 24, and 29 hours after the injection.

Although cortisone is administered on an individual basis rather than on a weight basis, the dosage in these schedules was moderately high. In human adults, 100 mg. per day are usually administered after high initial doses, even in acute conditions such as rheumatic fever.

## RESULTS

*Single Injection of 200 Milligrams of Cortisone into 1 Ewe.*—The results of this phase of the experiment are tabulated in table 1.

*Daily Injection of Cortisone for Seven Days.*—The normal percentage values for the hematocrit of ewes after six days' readings were found to be: sheep A, 43 ± 0; sheep B, 43 ± 1; sheep D, 44 ± 3; and sheep 304, 43 ± 2. After the administration of 400 mg. of cortisone, the hematocrit tended to lie in the lower limit or slightly below the preinjection range. On the fifth and sixth days of injections, sheep D had packed cell percentages of 33 and 36, respectively.

Except for the decrease in the numbers of red cells accompanying the low hematocrit reading, there was no significant change in the red cell count or in the hemoglobin percentage of the blood during the time cortisone was administered.

In animals A, D, and 304, there was a marked increase in the number of circulating leukocytes on the third day of injections. In animal A, there was an increase from a preinjection average of 7,500 to 12,250 cells per 1 cmm.; in animal D, there was a similar increase from 6,600 to 11,900 cells per 1 cmm., and in animal 304, the increase was from 5,900 to 9,000. In sheep D, the high number of leukocytes was maintained throughout the period of injections. In animals A and D, the leukocyte count fell to normal by the sixth day, but increased slightly under the influence of the double injections which occurred on the sixth and seventh days. Preinjection numbers were found in all animals two days after the cessation of injections.

There was a marked but very transitory shift in the percentage of circulating neutrophils in animals A, D, and 304. In sheep A, the circulating neutrophils rose from a preinjection percentage of 31 to 58; in sheep D, the rise was from 40 to 58 per cent, and in sheep 304, the rise was from 29 to 52 per cent. After the third day of cortisone injections, the percentage of neutrophils fell readily and was not influenced by the doubled injections on the sixth and seventh days. In sheep B, the neutrophil percentage increased only after the increase in cortisone injections on the sixth day. Under these conditions, the percentage rose from 32 to 50 on the first day after injections were discontinued.

There was a converse shift in the percentages of circulating lymphocytes. In sheep A, the percentage fell from a preinjection average of 63 to 40 on the third day of injection. In sheep D, the comparable percentages were 55 to 36 and, in sheep 304, 61 to 43. Sheep B again showed a delayed response, the drop in the number of lymphocytes occurring only after the cortisone dosage was doubled on the sixth and seventh days. The decrease was from 64 to 42 per cent.

No decrease was observed in the number of circulating eosinophils in this phase of the experiment. The preinjection percentage

TABLE 1—Effect of a Single 200-Milligram Injection of Cortisone on Blood Constituents in 1 Ewe

| | Preinjection | After cortisone | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3½ hr. | 5½ hr. | 12 hr. | 24 hr. | 29 hr. | 48 hr. |
| Whole blood glucose (mg. per per 100 cc.) | 27.0 | 47.0 | 50.0 | 63.0 | 46.0 | 47.0 | ..... |
| Plasma chloride (mEq. per liter) | 105.0 | 105.0 | 108.0 | 107.0 | 105.0 | ..... | ..... |
| Plasma sodium (mEq. per liter) | 143.0 | 143.0 | 142.0 | 152.0 | 146.0 | ..... | ..... |
| Plasma potassium (mEq. per liter) | 4.8 | 4.4 | 4.7 | 4.7 | 4.8 | 4.8 | ..... |
| Total leukocytes (per cmm.) | 6,850.0 | 8,750.0 | 6,000.0 | 10,000.0 | 6,850.0 | 11,400.0 | 8,450.0 |
| Neutrophils (%) | 30.0 | 50.0 | 47.0 | 56.0 | 54.0 | 39.0 | 37.0 |
| Lymphocytes (%) | 63.0 | 44.0 | 52.0 | 41.0 | 42.0 | 53.0 | 58.0 |
| Eosinophils (%) | 4.0 | 1.0 | 0.0 | 0.0 | 1.0 | 3.0 | 3.0 |

of 2 to 4 was found throughout the injection period. Five days after the cessation of injections, and for four days thereafter, the eosinophil percentage rose in 3 sheep to a range of 7 to 13.

The results obtained on blood and plasma analyses are summarized in table 2. The preinjection levels of whole blood glucose in the 4 ewes was found to be somewhat lower than accepted normal values. They are no lower, however, than values which we have obtained on other healthy sheep in the University of California flock.

A blood glucose increase was observed in 3 of the animals after cortisone had been administered for two days. The glycemic response of sheep A and B was most marked, the peak concentration occurring seventy-two hours after the first injection. In these 2 sheep, the glucose concentration tended to return to normal values but was increased after cortisone injections were doubled. The increase was most marked in sheep A. Sheep D showed less glycemic response, but the glucose concentration did reach a peak of double the normal concentration after cortisone injections were doubled. Sheep 304 showed no increase in glucose.

Since no urine was collected, there are no data on the urinary glucose. Glucose tolerance was not determined at any time during the experimental period.

The tabulated data show that plasma chloride was increased through the third day of injections and then decreased below preinjection levels. It was again retained on the seventh day, after the cortisone dosage was doubled.

The plasma potassium levels were elevated through the fourth day of injections in all animals. In sheep A and B, the level of potassium was again elevated as a result of increasing the cortisone dosage.

No retention or loss of sodium occurred. There was some day-to-day fluctuation in the sodium concentration before and after the administration of cortisone. Some of this variance might have been caused by the sensitivity of the flame spectrophotometer. Some variance was undoubtedly caused by a real day-to-day fluctuation in sodium concentration.

Both high and low plasma potassium levels can be detected by observing the T wave and the S-T segment of the electrocardiogram. It was for this reason that electrocardiograms were taken on the sheep. It was thought that the tracing would serve as an added check on the potassium analyses. Subsequent work, which has been done in this laboratory on the effect of high plasma potassium levels on the electrocardiogram of sheep, has indicated that the levels of potassium found in the animals on cortisone regimen were not high enough to influence the amplitude of the T wave or the configuration of the S-T segment.

## DISCUSSION

In considering the metabolic reaction to cortisone or other adrenal steroids, a cardinal point is the fact that no fixed amount of hormone represents a physiological dose. The needs of the tissues vary widely. A dose which may be pharmacological under optimal conditions may be less than optimal under conditions of stress and may not prevent hypocorticalism. Cortisone, therefore, is administered until a desired effect is achieved, but signs of a high degree of hypercorticalism are countered by withdrawal of the hormone.

During hypercorticalism, there is an increased mobilization of amino acids from the peripheral tissues. Lymphoid tissue is the most susceptible to the hypercortical state. Although not the sole factor controlling the release of lymphocytes into the peripheral blood stream, the adrenal cortex is one of the important ones. The lymphocytopenia which occurs during periods of stress is caused by the increased activity of the adrenal cortex. Adrenal steroids, such as cortisone, with an oxygen atom in position 11 are the adrenal constituents responsible for the lymphocyte shift. Because of this phenomenon, cortisone is being investigated for its therapeutic value in human cases of chronic or acute leukemia and of lymphosarcoma.[14]

The increase in neutrophils is also a common finding in cortisone-treated animals. The hormone has been used successfully in treating a case of profound agranulocytosis in a human subject.[2]

Our data show that the action of cortisone in releasing neutrophils and suppressing the lymphocytes is rapid in the ovine species. Detectable changes occurred in three and one-half hours after the administration of 200 mg. of the hormone; these shifts persisted for twenty-four hours. The significance of the increase in the number of total circulating leukocytes after a single injec-

tion is not known because no data are at hand to show the degree of diurnal variation in the leukocytes in sheep.

In sheep receiving cortisone injections each day for seven days, diurnal variation of leukocytes may be neglected because sampling was done at the same time each day. The increase in total circulating leukocytes in these animals is a real function of adrenocortical hormone activity.

The eosinophil response to cortisone varied with the condition of the experiment. The transitory eosinopenia seen in the sheep given a single injection was not seen in the other 4 animals on a daily injection schedule. This may have been due to the fact that blood samples in the latter sheep were not taken early in the course of injections. The marked eosinophilia found after the cessation of multiple injections has not been emphasized in other reports. Since adrenal steroids have a selective action on mesenchymal tissue, it is possible that the rise in eosinophil percentage was caused by a metaplasia of tissue fibroblasts. This phenomenon could be coupled with a blocking action of cortisone which prevented the eosinophil release until the hormone concentration was negligible.

The hematocrit values obtained in this study are at variance with those reported by Holman,[10] who found an average packed cell percentage of 30.5 and a range of 22.0 to 39.0 in 100 sheep over 3 months of age. Our values correspond more closely with those obtained by Becker and Smith,[1] who reported an average packed cell percentage of 37.9 ± 0.36 in 108 determinations on sheep blood. It is possible that the decrease in packed red cell volume on the fifth and sixth days of injections reflects the rather wide fluctuations which have been reported. In view of the electrolyte values obtained during these days, and the constancy of the electrocardiogram, a frank hydration of the animal was unlikely.

In nonruminants, the glycemic response to cortisone or other agent is determined on a fasting animal. Fasting levels in ruminants are obtained only after the animal has been off rations for three or four days. Hence, fasting blood sugar could not be determined in this experiment.

Experience in human subjects has shown that the effects of cortisone on carbohydrate metabolism may vary widely. Sprague *et al.*[20] reported that no significant change is usually found in the fasting blood glucose of cortisone-treated patients. In some cases where the individual is on a high dosage, a diabetes has been produced.[21] This has been characterized by glycosuria, ketonuria, and acidosis. In a few individuals, these signs become apparent after eight days of therapy.

The glucose response observed in the 1 sheep given a single 200-mg. injection was remarkably rapid and persisted for approximately twenty-four hours. The degree of hyperglycemia observed after a single 200-

TABLE 2—The Effect of Seven Daily Injections of Cortisone on Blood Glucose and Plasma Electrolytes of 4 Ewes

| | | Preinjection | | | | | | After cortisone injections | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 200 mg. | 200 mg. | 100 mg. | 100 mg. | 100 mg. | 200 mg. | 200 mg. | ..... | ..... | ..... |
| Whl. blood | A | 36.0 | 20.0 | 20.0 | 36.0 | 27.0 | 36.0 | .... | .... | 63.0 | 72.0 | 66.0 | 50.0 | 58.0 | 66.0 | 52.0 | 38.0 | 34.0 |
| glucose | B | 30.0 | 27.0 | 36.0 | 35.0 | 22.0 | 25.0 | .... | .... | 53.0 | 75.0 | 41.0 | 28.0 | 42.0 | 46.0 | 43.0 | 38.0 | 36.0 |
| (mg. per | D | 27.0 | 30.0 | 33.0 | 33.0 | 32.0 | 33.0 | .... | .... | 44.0 | 37.0 | 50.0 | 46.0 | 56.0 | 71.0 | 68.0 | 44.0 | 36.0 |
| 100 ml.) | 304 | 24.0 | 23.0 | 49.0 | 49.0 | 25.0 | 25.0 | .... | .... | 37.0 | 44.0 | 33.0 | 34.0 | 31.0 | 44.0 | 36.0 | 30.0 | 28.0 |
| Plasma | A | .... | 137.0 | 168.0 | 149.0 | 153.0 | 161.0 | .... | .... | 152.0 | 149.0 | 152.0 | 146.0 | 148.0 | 175.0 | 161.0 | 158.0 | .... |
| sodium | B | .... | 164.0 | 152.0 | 166.0 | 146.0 | 161.0 | .... | .... | 149.0 | 149.0 | 139.0 | 144.0 | 149.0 | 160.0 | 153.0 | 144.0 | .... |
| (mEq. per | D | .... | .... | 168.0 | 149.0 | 147.0 | 157.0 | .... | .... | 144.0 | 147.0 | 147.0 | 153.0 | 147.0 | 157 0 | 143.0 | 152.0 | .... |
| liter) | 304 | .... | 145.0 | 159.0 | 146.0 | 160.0 | 146.0 | .... | .... | 153.0 | 144.0 | 149.0 | 151.0 | 144.0 | 152.0 | 143.0 | 147.0 | .... |
| Plasma | A | 5.4 | 5.9 | 5.1 | 5.0 | 5.3 | 5.3 | .... | .... | 6.7 | 6.6 | 5.6 | 5.9 | 5.6 | 6.4 | 5.7 | 5.3 | .... |
| potassium | B | .... | 5.6 | 4.9 | 5.5 | 5.6 | .... | .... | .... | 6.2 | 6.4 | 5.2 | 5.3 | 5.7 | 6.1 | 5.6 | 5.1 | .... |
| (mEq. per | D | .... | 5.8 | 5.8 | 4.7 | 5.2 | 4.9 | .... | .... | 6.4 | 5.9 | 5.4 | 4.9 | 5.2 | 5.2 | 5.1 | 4.8 | .... |
| liter) | 304 | .... | 5.2 | 5.9 | 5.5 | 5.5 | 5.6 | .... | .... | 6.7 | 6.1 | 5.0 | 5.9 | 5.2 | 5.4 | 5.3 | 5.1 | .... |
| Plasma | A | 108.0 | 108.0 | 108.0 | 108.0 | 108.0 | 108.0 | .... | .... | 118.0 | 101.0 | 101.0 | 101.0 | 109.0 | 101.0 | 109.0 | 107.0 | .... |
| chloride | B | 108.0 | 108.0 | 106.0 | 107.0 | 105.0 | 108.0 | .... | .... | 118.0 | 101.0 | 101.0 | 99.5 | 108.0 | 105.0 | 107.0 | 108.0 | .... |
| (mEq. per | D | .... | 108.0 | 108.0 | 104.0 | 107.0 | 108.0 | .... | .... | 110.0 | 101.0 | 104.0 | 101.0 | 110.0 | 104.0 | 107.0 | 108.0 | .... |
| liter) | 304 | .... | 109.5 | 109.5 | 109.5 | 109.5 | 109.5 | .... | .... | 113.0 | 107.0 | 104.0 | 107.0 | 115.0 | 110.0 | 110.0 | 109.0 | .... |

mg. injection of cortisone was the same as that found after 4 animals had received 400 mg. each. This would imply that the glycemic response did have a limit and that if cortisone were to be used for a relatively short and moderate hyperglycemic effect a single large dose might suffice. However, 1 of the 4 animals on the multiple injection regimen did not respond at any time, and the time relations of the response in the remaining 3 sheep did not correspond with one another. These factors should be taken into account in interpreting any glucose levels obtained with cortisone, and they reemphasize the point that the hormone should be administered on an individual response basis.

The mobilization of ions in a cortisone-treated subject may vary considerably, depending upon the dosage and the duration of therapy. Cortisone, at 200 mg. per day, may cause sodium retention in the human subject[6, 8] or, if treatment is prolonged, it may cause sodium excretion and loss.[20] Plasma potassium and chloride levels frequently fall after five or six weeks of treatment. This fall reflects an intracellular potassium deficit and produces a hypopotassemic, hypochloremic alkalosis.

The data which we obtained on the sheep receiving multiple injections agree with those obtained in the human subject receiving 100 mg. of cortisone per day. The transitory shifts in potassium and chloride observed in our experiment indicated the possible direction of ultimate shift, but they were not severe enough to produce an acidosis or an alkalosis.

## SUMMARY

When a single dose of 200 mg. of cortisone acetate (cortone acetate®) was injected into 1 ewe, a hyperglycemia resulted after three and one-half hours and persisted for twenty-four hours. There was no change in the plasma electrolytes. There was no significant increase in the total number of circulating leukocytes, but there was an increase in the percentage of neutrophils and a decrease in the percentage of lymphocytes. A transitory eosinopenia was observed.

When cortisone was administered to 4 sheep for seven consecutive days, the glycemic response was more sustained in 3 of the animals. One ewe did not respond. A transient increase was noted in the plasma potassium and chloride; this was followed by a decrease in concentration to below preinjection levels before the return to the normal range. No change was observed in sodium concentration. There was an increase in the number of total circulating leukocytes, an increase in the percentage of neutrophils, and a decrease in the percentage of lymphocytes. A postinjection eosinophilia persisted for four days.

There was considerable variation in the degree and the time of maximal metabolic response among the 4 ewes.

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# Studies on the Pathogenicity of Brucella Abortus for Swine. I

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## Review of Literature

For many years, most investigators have believed that swine were rarely, if ever, naturally infected with *Brucella abortus*. Huddleson[1] stated, in 1943, "No one has reported the isolation of *Br. abortus* from naturally infected swine." Graham *et al.*,[2] in 1930, fed milk from 10 Brucella-infected cows to 7 pregnant gilts for approximately ten weeks but failed to produce evidence of infection by this means. Gilman and co-workers,[3] in 1934, were likewise unable to infect hogs experimentally with *Br. abortus*. Later, in 1949, McCullough and Eisele[4] reported the recovery of *Br. abortus* and *Brucella melitensis* from swine carcasses sampled at random in a Chicago packing plant. These recoveries were from 8 naturally infected swine which came from different sources. Borts, McNutt, and Jordan[5] isolated *Br. melitensis* from 12 hogs in Iowa in 1946.

Studies of Brucella interspecies transmissibility at the Purdue University Agricultural Experiment Station,[6] in 1947, have shown that *Brucella suis* infection was readily induced in cattle by artificial exposure through the teat canal.

Manthei[7] reported that *Br. melitensis* was recovered from 2 of 6 heifers placed in contact with 26 goats naturally infected with *Br. melitensis*. Taylor *et al.*[8] indicated that *Br. melitensis* infection was found as a naturally occurring disease of cattle in France.

Thus, it has been shown that all of the recognized types of Brucella (*abortus*, *suis*, and *melitensis*) have been isolated from both swine and cattle.

If natural *Br. abortus* infection occurs commonly or even occasionally in swine, its importance could scarcely be overemphasized. Not only would this possibly be of economic importance to the swine industry, but the hazard of spread from carrier swine to cattle likely would present an additional problem in the control of the disease in cattle. Also, if swine commonly serve as a reservoir of *Br. abortus* infection, it would be an additional hazard to human health for swine raisers, handlers, and particularly butchers. This would be true regardless of whether or not *Br. abortus* infection in swine resulted in recognizable clinical disease.

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## Experimental Procedure

In the studies reported here, efforts were directed toward transmitting *Br. abortus* infection from cattle to swine by feeding infected cow's milk to swine. The infected cow's milk used was obtained from 6 dairy cows which had been successfully exposed artificially to *Br. abortus*. Such infected milk was fed to a total of 22 swine, three to five months old. Four separate trials were conducted. All of the experimental animals, both cows and pigs, were free from brucellosis, so far as could be determined by standard methods, at the time of entering this experiment.

In the first trial, an open Guernsey cow was exposed to two laboratory strains (56 and 3003) of *Br. abortus* by injecting 6 cc. of a living suspension (adjusted turbidity to No. 1 McFarland's nephelometer) into the right front and right rear quarters of the udder. After infection had been established in the udder, as evidenced by repeated isolations of living *Br. abortus* from the milk, it was fed to 5 pigs for approximately three months. The cow and the pigs were kept in a small outside lot and were fed and watered from the same containers. During this feeding period, milk samples from the cow were examined three times weekly. These examinations showed that this cow continued to shed viable *Br. abortus* in the milk during this period.

Individual blood samples were obtained from all animals at weekly intervals. Such blood samples were examined for Brucella organisms by direct culture methods, using tryptose-agar (Difco) plates incubated under 10 per cent $CO_2$ at 37 C. Contaminated samples were injected into guinea pigs which were killed after thirty days and examined for Brucella organisms. The agglutination responses of blood samples were determined by the rapid plate-agglutination test as described by Huddleson,[1] using plate antigen as prepared by United States Bureau of Animal Industry at serum dilutions 1 : 25, 1 : 50, 1 : 100, 1 : 200, and 1 : 400.

At the termination of the feeding period, the pigs were brought to autopsy for bacteriological examinations. The tissues examined were popliteal, prefemoral, inguinal, iliac, mesenteric, bronchial, gastrohepatic, cervical, mandibular, and suprapharyngeal lymph nodes, and liver, spleen, kidney, heart, and lung. These swine tissues were plated directly on tryptose agar (Difco) and composites of tissue triturates

were injected into guinea pigs. The guinea pigs were brought to autopsy after thirty days and their tissues examined for Brucella organisms as above.

In the second trial, 1 nonpregnant cow and 5, 3-month-old pigs were used. The methods employed were the same as those described above except as follows: (1) The cow and pigs were not kept in the same pen. (2) The period of feeding Brucella-infected milk to the pigs was five months. (3) At the end of the trial period, the cow, as well as the swine, were brought to autopsy for bacteriological examination.

A third trial was conducted using 2 cows and 6 pigs. The methods used were the same as above except as follows: (1) One of the cows used was not pregnant and the other was in the fifth month of pregnancy when exposed. (2) The cows were exposed using two different strains of *Br. abortus*. One strain was recovered from the milk of the cow used in the second trial. The other strain had been recovered from the submaxillary lymph node of a hog cultured at a large Midwestern slaughtering establishment. (3) The cows and pigs were housed together in a barn for one month and, during the latter period of the trial, the cows were kept in a small outside pen adjoining the barn. (4) Beginning at the end of the first month after initiation of milk feeding, 1 pig was brought to autopsy each successive week, except for a two-week interval between the last two autopsies.

In a fourth trial, 2 cows and 6 swine were used. The methods used were similar to those of the previous trials except as follows: (1) One cow was exposed to the strain of *Br. abortus* which had been recovered from the suprapharyngeal lymph node of a hog in the previous trial, and the other to a strain of *Br. abortus* which had been recovered from the submaxillary lymph node of a hog examined at a large commercial slaughtering establishment. (2) The suspension of *Br. abortus* was injected into all four quarters. (3) One cow was exposed to approximately 1,140,000,000 organisms per quarter and the other to approximately 6,200,000,000 organisms per quarter. (4) Autopsy examinations were conducted seven weeks after institution of the trial. (5) Blood samples were drawn twice weekly.

## EXPERIMENTAL RESULTS

*First Trial (Table 1).*—No high, definitely positive blood serum-agglutination titers were observed in any of these 5 pigs, even though Brucella organisms were recovered five times from the blood of 1 pig (No. 3326) and from the tissues of this one and from the tissues of 2 others (No. 3322 and 3328 at autopsy. The cultures obtained conformed to the characteristics of *Br. suis*. Repeated cultures of the cow's milk used to feed these pigs gave *Br. abortus* only, not *Br. suis*.

TABLE 1—Results of Serum-Agglutination and Blood Cultural Examinations of Swine Fed Brucella Abortus-Infected Cow's Milk

| Pig (No.) | Agglutination response, days after initiation of milk feeding | | | | | | | | Autopsy cultural results for Brucella |
|---|---|---|---|---|---|---|---|---|---|
| | 20 | 23 | 30 | 37 | 41 | 51 | 58 | 65 | |
| 3322 | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | Positive* |
| 3324 | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | Negative |
| 3326 | T – – – – | T – – – – | PPT – – | PPT – – | PT – – –* | PT – – –* | + P – – –* | PT – – –* | Positive* |
| 3328 | – – – – – | T – – – – | T – – – – | T – – – – | – – – – – | – – – – – | – – – – – | – – – – – | Positive* |
| 3330 | – – – – – | – – – – – | T – – – – | T – – – – | T – – – – | T – – – – | – – – – – | – – – – – | Negative |

*Recovery of *Brucella suis*. + = complete agglutination. P = partial agglutination. T = trace of agglutination. – = negative.

Second Trial (Table 2).—Only slight, transient blood serum-agglutination titers were elicited in 3 of these pigs, the remaining 2 being entirely negative.

Brucella organisms were not recovered from any of these pigs. *Brucella abortus* was

TABLE 2—Results of Serum-Agglutination and Blood Cultural Examinations of Swine Fed Brucella Abortus-Infected Cow's Milk

| Pig (No.) | Agglutination response, days after initiation of milk feeding | | | | | | | | | Autopsy cultural results for Brucella |
|---|---|---|---|---|---|---|---|---|---|---|
| | 7 | 27 | 33 | 41 | 55 | 61 | 76 | 97 | 110 | |
| 3167 | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | Negative |
| 3169 | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | Negative |
| 3171 | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | T – – – – | – – – – – | – – – – – | – – – – – | Negative |
| 3186 | – – – – – | +P – – – | PP – – – | T – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | Negative |
| 3188 | – – – – – | T – – – – | T – – – – | T – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | Negative |

+ = complete agglutination. P = partial agglutination. T = trace of agglutination. – = negative.

recovered repeatedly from the cow's milk fed to these pigs.

*Third Trial (Table 3).*—More pronounced and persistent blood serum-agglutination titers were observed than in the previous two groups of pigs. In 3 pigs, diagnostic titers were recorded for the first time on the fifty-fifth day after the feeding of infected milk was started. The 3 other pigs had been slaughtered with negative titers prior to the fifty-fifth day.

*Brucella abortus* was recovered repeatedly during the period of the experiment from the milk of the 2 cows. One of these cows aborted during her seventh month of pregnancy, and *Br. abortus* was recovered from both the fetus and placenta. *Brucella abortus* was recovered from the blood of this cow intermittently during the period of this experiment and, at autopsy, from the spleen, lung, uterus, udder, and the prefemoral, popliteal, mesenteric, bronchial, and supramammary lymph nodes.

Brucella organisms were recovered, at autopsy, from 2 of the 6 pigs in this group. One of these cultures (from the suprapharyngeal and middle cervical lymph nodes of pig 112) typed as *Br. abortus*, but the other (from pig 120) typed as *Br. suis* and was obtained from four different lymph nodes, *i.e.*, superficial inguinal, bronchial, mandibular, and gastrohepatic.

*Fourth Trial (Table 4).*—Blood serum-agglutination titers indicative of infection were not observed in this group of swine. Brucella organisms were not recovered from the blood of the swine during the course of the trial. Autopsy failed to reveal the presence of living Brucella in any of the swine carcasses. Both cows became infected following exposure, as measured by the blood serum-agglutination response and recovery of Brucella. One cow died during the second week of the trial. Living *Br. abortus* was recovered from the milk of each of the four quarters of this cow up until the time of death and from the milk of the four quarters of the other cow throughout the milk-feeding period. *Brucella abortus* was recovered from the blood stream of the latter cow on the thirtieth day after exposure. At autopsy, this animal revealed the presence of living *Br. abortus* in the spleen, bladder, udder, and in the prefemoral, popliteal, internal and external iliac, mesenteric, bronchial, gastrohepatic, cervical, mandibular, and suprapharyngeal lymph nodes.

TABLE 3—Results of Serum-Agglutination and Blood Cultural Examinations of Swine Fed Brucella Abortus-Infected Cow's Milk

| Pig (No.) | Agglutination response, days after initiation of milk feeding | | | | | | | | Autopsy cultural results for Brucella |
|---|---|---|---|---|---|---|---|---|---|
| | 15 | 34 | 41 | 48 | 55 | 62 | 69 | 76 | |
| 112 | – – – – – | – – – – – | – – – – – | – – – – – | +PT – – | * | ..... | ..... | Pos. (*Br. abortus*) |
| 120 | – – – – – | – – – – – | – – – – – | – – – – – | +PT – – | +PT – – | PTT – – | PT – – – * | Pos. (*Br. suis*) |
| 136 | – – – – – | – – – – – | – – – – – | * | ..... | ..... | ..... | ..... | Neg. |
| 148 | – – – – – | – – – – – | – – – – – | – – – – – | + +PT – | +PT – – * | ..... | ..... | Neg. |
| 277 | – – – – – | – – – – – | – – – – – | – – – – – | * | ..... | ..... | ..... | Neg. |
| 144 | – – – – – | * | ..... | ..... | ..... | ..... | ..... | ..... | Neg. |

*Removed for slaughter. P = partial agglutination. T = trace of agglutination. + = complete agglutination. — = negative.

## DISCUSSION

In these trials of feeding *Br. abortus*-infected cow's milk to pigs, organisms which typed as *Br. suis* were recovered from 4 of a total of 22 pigs. This raises at least three questions which can not be answered satisfactorily at this time.

1) Were these pigs exposed to an unde-

TABLE 4—Results of Serum-Agglutination and Blood Cultural Examinations of Swine Fed Brucella Abortus-Infected Cow's Milk

| Pig (No.) | Agglutination response, days after initiation of milk feeding | | | | | | | Autopsy cultural results for Brucella |
|---|---|---|---|---|---|---|---|---|
| | 7 | 13 | 20 | 27 | 35 | 41 | 43 | |
| 830 | – – – – – | – – – – – | – – – – –* | ..... | ..... | ..... | ..... | Negative |
| 832 | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | T – – – | Negative |
| 834 | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | PT – – – | Negative |
| 836 | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | Negative |
| 838 | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | Negative |
| 840 | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | – – – – – | Negative |

*Died. P = partial agglutination. T = trace of agglutination. — = negative.

termined source of *Br. suis* as well as the intentional exposure to *Br. abortus*? This possibility certainly can not be denied, particularly at this Station where many experiments with brucellosis in swine have been conducted during the past eight years. However, with the precautions that were taken to avoid such exposure to *Br. suis*, we must consider the possibility that this did not occur.

2) Is it possible, under proper conditions, for the cultural characteristics of *Br. abortus* to be altered to those of *Br. suis* by living and multiplying in swine tissues? Some other of our experimental evidence also raises this question, but the evidence, as yet, is not sufficient to justify any conclusion.

3) Are present techniques for typing Brucella organisms sufficiently precise to justify current common usage for identifying the three types of Brucella, *i.e., abortus, suis,* and *melitensis.*

Certainly, further research is warranted and is in progress to attempt to answer these and other questions.

## SUMMARY

1) Infectivity trials using *Brucella abortus*-infected cow's milk fed to susceptible pigs indicated that, under these conditions, *Br. abortus* was not highly pathogenic for swine but that some infections were experimentally induced.

2) *Brucella suis* isolations reported here from pigs fed *Br. abortus*-infected cow's milk raise questions which can not be answered satisfactorily at this time. (*See* Discussion.)

3) These and other experiments previously reported,[6] using *Br. suis* as exposure material for cattle, indicate the need for further research on the interspecies transmissibility of Brucella organisms.

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# The Bacteriological Aspects of Experimental Brucellosis in Dogs Following Oral Exposure. II. Effects of Feeding Brucella-Infected Milk to Young Dogs

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EXPERIMENTAL BRUCELLA infections can be induced in dogs following oral inoculation of *Brucella abortus* cultures, as has been shown by van der Hoeden[4, 5] and by Feldman, Bollman, and Olson.[1] A single feeding of aborted fetuses and placentas from Brucella-infected cows may also produce the disease in dogs.[3] Since the ingestion of infected tissues may induce brucellosis in the dog, it was deemed advisable to determine the effect of feeding milk from cows which contained Brucella organisms and agglutinins. The following report deals with the continuous, *ad libitum* feeding of such milk.

## MATERIALS AND METHODS

The milk from 4 cows which were shedding *Br. abortus* USDA strain 2308 from one or two quarters was pooled and fed to the dogs. As determined by plate counts, the cream of the positive quarter samples contained from 90 to 3,000 Brucella per milliliter. The agglutinin titer of the samples ranged from 0 to 1 : 400. The control animals were fed milk which did not contain Brucella or Brucella agglutinins.

The tube-agglutination test, starting with the 1 : 2 dilution of serum, was used making serial twofold dilutions. Prior to the start of the trials, none of the serums contained demonstrable agglutinins. Serum-agglutination tests were conducted at weekly intervals after the consumption of milk was commenced.

Nineteen puppies, approximately 9 weeks old, were used in the experiment. Dogs 1 to 6 and 15 to 19 were the progeny of 2 females whose serums contained no demonstrable Brucella agglutinins. Dogs 7 to 13 and dog 14 were the young of 2 females, respectively, (No. 6 and 8 of a previous experiment[3]) which were harboring Brucella in their bronchial-cervical lymph nodes, as determined by culture of material collected at autopsy. The serums of both dams contained Brucella agglutinins.

Dogs 1 to 3, 5, 7 to 10, and 13 to 18 were fed the Brucella-infected milk *ad libitum*. Dogs 4, 6, 11, 12, and 19, which were fed milk from a healthy cow, served as controls. Anal swabs were taken for bacteriological examinations on the sixty-fifth day and at weekly intervals thereafter. One or more of the dogs from the two groups were killed* and examined at 43, 62, 66, 69, 91, 99, 105, and 112 days. Milk was withheld one to five days prior to death, in some instances, while some dogs were given free access to the milk until the time of autopsy. The following materials were subjected to bacteriological examination: blood, urine, femoral bone marrow, homogenates of tonsil and of bronchial-cervical, popliteal-prescapular, mesenteric, and gastrohepatic-iliac lymph nodes; the homogenized visceral pool consisting of the spleen, liver, kidney, genital tract, and lung was also examined for the presence of Brucella. The techniques for isolation of *Br. abortus* from these excreta or tissues have been described previously.[3]

## RESULTS

None of the serums from the dogs showed demonstrable Brucella agglutinins during the course of the experiment. Brucella were not recovered from the anal swabbings. Brucella were not isolated from any of the tissues or excreta secured from the dogs at autopsy. The time of death, duration of milk-free diet prior to autopsy, and other data are summarized in table 1.

## DISCUSSION

Since some of the young dogs were the progeny of Brucella-infected dams while others were whelped from noninfected females, it was considered desirable to compare the susceptibility and agglutinin response of the two groups. Both groups were equally resistant and the agglutinin response was lacking in all; hence, comparative data were not secured. Apparently, Brucella were not present in the milk in sufficient numbers to produce canine infection.

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*To accomplish this, the dogs were completely anesthetized by intravenous injection of nembutal ® and then exsanguinated by severing the femoral artery.

Karlson and Clausen[2] have reported a spontaneous case of brucellosis in a dog. When the animal was presented to these investigators, the serum-agglutination test was positive at 1 : 50 and incomplete at the 1 : 100 level. The owner reported that for several years the animal had consumed daily a quart of milk from Brucella-infected cows. At the laboratory, meat and dog biscuits were fed for three months, and a quart of milk known to contain *Br. abortus* was added to the diet daily for the following two months. Employing guinea pig inoculations, 14 attempts to recover Brucella from the blood were made during the time milk was ingested. Brucella were present in a 2-ml. blood sample drawn at one month after the first feeding of positive milk. The serum agglutinin titer did not change, and *Br. abortus* was not recovered from the tonsils, lymph nodes, or viscera at the termination of the five-month observation period.

TABLE I—Results of Ad Libitum Feeding of Brucella-Infected Milk to Young Dogs

| Individual dog | Days from initial feeding to death | Days milk withheld prior to autopsy | Serum agglutinins for Brucella | Bacteriological examinations for Brucella of excreta and tissues |
|---|---|---|---|---|
| 5, 13, 18 | 43 | 4 | none | negative |
| 8, 14, 11* | 62 | 2 | none | negative |
| 19* | 66 | 1 | none | negative |
| 2, 6*, 15 | 69 | none | none | negative |
| 9, 10, 12* | 91 | none | none | negative |
| 1, 3 | 99 | none | none | negative |
| 7, 17 | 105 | none | none | negative |
| 4*, 16 | 112 | none | none | negative |

*Controls—fed milk which contained no Brucella or agglutinins.

The *Br. abortus* culture recovered from the animal's blood by guinea pig inoculation probably represented those derived from the infected milk which was being consumed at that time. The dog, however, apparently was infected or had been infected, as demonstrated by the agglutination test, prior to experimentation. The animal had lived on a farm for several years on which 9 of 14 cattle were reactors. This fact would not preclude the possibility that the original infection might have been induced by the ingestion of aborted fetal or of placental material. Such tissues, following a single oral exposure, have been demonstrated[3] to be a potent source of infection for the dog.

While Brucella-infected milk may be responsible for brucellosis in man, it is believed that such milk may rarely be incriminated as a source material for the disease in dogs. Evidently, Brucella are not generally present in sufficient numbers in the milk of infected cows to produce the disease in dogs. It is probable, therefore, that the majority of canine *Br. abortus* infections and resultant agglutinin titers are due to the ingestion of infected tissues and not the consumption of milk which contains the organism.

## SUMMARY

Fourteen young dogs were continuously fed, *ad libitum*, milk which contained *Brucella abortus* strain 2308 and Brucella agglutinins. Animals from the group were killed and autopsies performed at 43, 62, 69, 91, 99, 105, and 112 days, respectively. During the course of the investigations, demonstrable serum agglutinins for Brucella were not detected. Attempts to isolate Brucella from excreta and tissues failed.

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# Studies on a Pasteurella Multocida Chicken Embryo Vaccine. II. Type-Specific Nature of Immunity Elicited by a Monovalent Pasteurella Multocida Vaccine

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IN THE FIRST of these studies,[1] it was shown that two inoculations of a *Pasteurella multocida* chicken embryo vaccine protected mice against more than 1,000 lethal doses of the homologous organisms. It was the purpose of this study to confirm and reëmphasize the importance of selecting *P. multocida* strains for bacterin and antiserum production on a serological or immunological basis rather than on zoölogical grounds.

## Literature Review

It would serve no useful purpose here to review all of the literature extant on the serological and antigenic nature of *P. multocida*. This has been done comprehensively by Rosenbusch.[2] The reports cited below have been dealt with in some detail because, for the most part, they confirm and clarify the results obtained by earlier workers.

The observations of Rosenbusch and Merchant,[3] in connection with the serological nature of the hemorrhagic septicemia Pasteurella organisms, were in agreement with the findings of a number of earlier workers. Their conclusions were briefly as follows: 1) Two distinct biochemical and serological species were found. One species was designated *P. multocida* and the other *Pasteurella hemolytica*. (2) The *P. multocida* strains could be divided into two principal groups on the basis of agglutination. (3) A definite correlation was found to exist between xylose and arabinose fermentation and agglutination relationships.

Little and Lyon,[4] employing a slide-agglutination technique with serums prepared in rabbits, divided their 30 strains of *P. multocida* into three distinct groups. It was also observed by Little and Lyon that the protective property of therapeutic antiserums was correlated with the type specificity of their *P. multocida* strains.

Roberts,[7] employing immune serums and mouse-protection tests, was able to distinguish four distinct types on the basis of immunological differences. He believed that among the 37 strains examined there were probably in all seven or eight types. Rosenbusch and Merchant list seven serological variants, while Roberts observed five strains that appeared to be immunologically distinct from his four main types.

The author[2] has demonstrated a type-specific soluble antigen associated with the capsule of *P. multocida* and, on the basis of this antigen, has typed members of the group by a precipitin reaction and a capsular-swelling technique. For a number of reasons, discussed at length in the above referred to study, an alphabetical designation was proposed and it is this which is used to identify the cultures employed in this investigation.

## Materials and Methods

The *P. multocida* strains used in this experiment were No. 1 (type A-bovine), No. 2 (type B-buffalo), and No. 3 (type C-canine). Information relative to the origin and maintenance of cultures, fertile eggs, experimental mice, and preparation of chicken embryo vaccine, including purity and sterility tests, will be found in the first of these studies.[1]

*Immunization.*—Data relative to the numbers of mice vaccinated and the type identity of the vaccine are given in table 1. Each mouse in the immunized groups received two doses of 0.5 ml. of vaccine intraperitoneally. The interval between doses was seven days. All mice were challenged fourteen days after the second inoculation.

*Method of Challenge.*—The challenge cultures and the dilutions of these cultures were prepared as outlined previously.[1] The volume of the challenge inoculation was 0.5 ml. and it was administered intraperitoneally. In order to obtain a suspension of highly virulent organisms for challenge, allantoic fluid from

A part of this study was taken from a thesis submitted to the Graduate School of Iowa State College in partial fulfillment of the requirements for the degree of master of science. The remainder was completed at the Division of Animal Pathology, Animal Diseases Research Institute, Hull, Quebec.

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third passage embryo cultures was employed in preference to broth cultures. The optimum incubation period for the chicken embryo cultures for vaccine production and challenge purposes was found to be approximately twenty-four hours. Only deaths occurring within seven days of challenge were recorded. Twenty-five per cent of the dead mice in all groups were cultured. Presumptive identification was carried out in the manner previously outlined.[1]

The methods and procedure employed in the experiment, the results of which are summarized in table 2, were similar to those just described. The culture employed was a highly virulent strain recovered from an acute outbreak of fowl cholera.

## RESULTS

The data obtained from the vaccination trials are summarized in table 1. The number of mice dying in each dilution group challenged is recorded. The vaccinated animals manifested a marked resistance to homologous challenge but displayed only a low degree of immunity to heterologous challenge.

During the course of this investigation, the question of local immunity of the peritoneum was raised. An experiment was designed to demonstrate that the immunity produced by the chicken embryo vaccine was of a general rather than local nature.

That such was the case is clear from the results summarized in table 2.

## DISCUSSION

Although an appreciable degree of cross immunity between types was not demonstrated in the foregoing experiment, it should not be concluded that cross protection is not sometimes observed. It is, however, usually of a low order and has been referred to by Roberts.[7] Cross-protection factors become less evident when the cultures employed are in a highly virulent state, as was the case in the experiment summarized in table 1.

The vaccination trials described have confirmed the results of the first of these studies[1] in regard to the immunogenic potency of a *P. multocida* chicken embryo vaccine. The marked immunogenicity of the chicken embryo vaccine would appear to be due to the fact that it contains large numbers of virulent capsulated organisms. The correlation of capsulation with virulence and immunogenicity has been demonstrated by Priestley.[5, 6] The author has demonstrated large amounts of capsular substance on organisms from chicken embryo cultures and has brought forward considerable evidence[2] that this capsular antigen is a type-specific soluble polysaccharide.

**TABLE 1—Type-Specific Nature of Immunity Produced by a Monovalent Pasteurella Multocida Vaccine in Mice to Intraperitoneal Challenge**

| Vaccinated with | Challenged with | Deaths per challenge-dilution groups | | | | |
|---|---|---|---|---|---|---|
| | | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | Totals |
| Type A | Type A | 0*/4** | 0/4 | 0/4 | 1/4 | 1/16 |
| | Type B | 4/4 | 4/4 | 4/4 | 2/4 | 14/16 |
| | Type C | 4/4 | 4/4 | 4/4 | 2/4 | 14/16 |
| Type B | Type A | 4/4 | 4/4 | 3/4 | 1/4 | 12/16 |
| | Type B | 1/4 | 0/4 | 1/4 | 2/4 | 4/16 |
| | Type C | 4/4 | 4/4 | 3/4 | 3/4 | 14/16 |
| Type C | Type A | 4/4 | 4/4 | 3/4 | 1/4 | 12/16 |
| | Type B | 4/4 | 4/4 | 4/4 | 3/4 | 15/16 |
| | Type C | 2/4 | 0/4 | 0/4 | 2/4 | 4/16 |
| Controls | Type A | ... | 4/4 | 4/4 | 3/4 | 15/16 |
| | Type B | ... | 4/4 | 4/4 | 3/4 | 15/16 |
| | Type C | ... | 4/4 | 4/4 | 4/4 | 16/16 |

*No. dead; **No. challenged.

It is obvious, considering the results of this study and those conducted by Little and Lyon[4] and Roberts,[7] that *P. multocida* strains for the production of bacterins and antiserums should be chosen on a serological or immunological basis rather than on zoölogical grounds. Information relative to the incidence of the various serological types in pasteurellosis of the domesticated species is urgently needed.

The following suggestions in connection with the preparation of *P. multocida*-immunizing agents are based upon the present study, the first study in this series,[1] and a report which is now in press.[2]

1) Bacterins and antiserums for administration to a given animal species should be prepared from those serological types most commonly recovered from pasteurellosis in that species.

2) Freshly isolated virulent strains showing less tendency toward dissocia-

**TABLE 2—Comparison of Intraperitoneal and Subcutaneous Routes of Challenge**

| | Deaths per challenge-dilution groups | | | | | |
|---|---|---|---|---|---|---|
| | $10^{-6}$ | | $10^{-8}$ | | $10^{-9}$ | |
| | i.p. | s.c. | i.p. | s.c. | i.p. | s.c. |
| Chicken embryo vaccine administered i.p. | 2/10 | 0/10 | 0/10 | 0/10 | ... | ... |
| Controls | 5/5 | 5/5 | 5/5 | 5/5 | 3/5 | 5/5 |

tion (blue colonial variants and flocculation in broth[10]) than old, degenerate stock cultures should be employed as seed cultures.

3) The incubation period of broth cultures should be terminated at the point of maximum capsulation.[3, 6]

4) Bacterin cultures may be sterilized satisfactorily by the addition of 0.25 per cent formalin rather than the customary 0.5 per cent amount.

It is planned to make available to laboratories in the near future cultures of *P. multocida* representative of the principal serological types.

## SUMMARY

Mice immunized with chicken embryo vaccines prepared from types A, B, and C of *Pasteurella multocida* demonstrated a strong resistance to homologous challenges, but displayed only a low degree of immunity to heterologous challenges.

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# The Propagation of the Causative Agent of Infectious Sinusitis of the Turkey in the Chicken Embryo

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INFECTIOUS SINUSITIS has been recognized as a disease entity of the turkey for many years. The first accurate description was made by Dodd[3] in England. Tyzzer[14] was the first to describe the disease in this country. The term infectious sinusitis was proposed by Dickinson and Hinshaw[2] to differentiate this disease from an inflammation of the sinuses observed in vitamin A-deficient turkeys.

The infectious nature of sinusitis of turkeys was demonstrated through transmission experiments.[4, 7, 10, 14] The propagation of the causative agent in chicken embryos has been reported.[6, 8, 9, 11, 12] Groupé and Winn[5] described growth characteristics of the agent that they isolated and propagated in chicken embryos.

## MATERIALS AND METHODS

The work reported here was designed to determine the most desirable procedures for the isolation of the causative agent of infectious sinusitis of the turkey and to provide further methods for study of the organism. Isolations of the agent were made from sinus exudate or air sac exudate of infected turkeys. The material was inoculated into the yolk sac of 6- or 7-day-old chicken embryos. All inoculums were checked for bacteriological sterility before isolations were attempted. Five isolations of the agent were made from sinus exudate of infected birds obtained from widely separated flocks and one isolation was made from the abdominal air sac exudate of 1 of the affected birds. The strains isolated were designated B, C, O, I, S, and Sa. Strain Sa was isolated from air sac exudate of the turkey from which the S strain was isolated. All detailed investigations were made by using the B strain, and general observations of the behavior of the other strains were compared to it.

All inoculations were made by standard procedures as described by Cunningham.[1] Virus concentrations in the materials tested were determined by the method of Reed and Muench[13] and are expressed as the lethal dose$_{50}$ (l.d.$_{50}$) doses per milliliter. The eggs used were obtained from a commercial poultry flock of Leghorn breeding. The eggs were embryonated in a small, forced-draft egg incubator and held in a similar machine after inoculation at 99 to 100 F. Most of the embryos were inoculated after seven days' incubation and were candled twice daily for the following twelve days. All embryo deaths occurring within forty-eight hours after inoculation were considered to be the result of injury from inoculation and the eggs were discarded.

On original isolation, it was found that the embryos died between the eighth and thirteenth day after inoculation. Serial transfers using 1 : 5 suspensions of yolk sac membrane in veal-infusion broth resulted in earlier deaths of all the embryos. The fifth serial passage caused a 100 per cent mortality by the fifth day, and further passages increased the percentage of deaths occurring the third and fourth days after inoculation.

The early passages of the agent in chicken embryos did not produce any gross lesions. After 15 serial passages of the agent, macroscopic subcutaneous hemorrhages were noted. These were very extensive and prominent by the twentieth and in all subsequent passages. All of the strains studied have produced similar macroscopic lesions in the chicken embryo.

*Comparative Efficacy of Various Routes of Inoculation and Comparative Concentrations of Infective Agent in Various Portions of the Infected Embryo.*—For these studies, the materials used were collected and pooled from each of five embryos which had died as a result of infection with the agent. These embryos had been stored from one to six days from the time of death until the time of harvesting at a temperature ranging between 6 and 8 C.

The routes of inoculation employed were on to the chorioallantoic membrane, into the allantoic chamber, and into the yolk.

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Titrations of the concentration of agent in yolk sac membrane, yolk, chorioallantoic membrane, and allantoic fluid were attempted by each of the above routes of inoculation using 0.2 ml. of inoculum per embryo. The yolk sac and chorioallantoic membranes were washed twice in sterile veal-infusion broth to remove the embryo fluids. Dilutions, based on their net washed weight, were made. The yolk and allantoic fluids were diluted volumetrically.

TABLE 1—Deaths Between the Third and Twelfth Days After Inoculation on to the Chorioallantoic Membrane of 0.2 Milliliters Each of Tenfold Dilutions of Various Portions of Embryos Infected with the Sinusitis Agent

| Dilution | Day of death 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Final mortality |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pooled yolk sac membrane | | | | | | | | | | | |
| $10^{-1}$ | | | | | | | | | | | 0/5 |
| $10^{-2}$ | | | | | | | | | | | 0/5 |
| $10^{-3}$ | | | | | | | | | | 1 | 1/5 |
| $10^{-4}$ | | | | | | | | | | | 0/5 |
| $10^{-5}$ | | | | | | | | | | | 0/5 |
| $10^{-6}$ | | | | | | | | | | | 0/5 |
| Pooled yolk | | | | | | | | | | | |
| $10^{-1}$ | | | | | | | | | 1 | | 1/5 |
| $10^{-2}$ | | | | 1 | 1 | | | | | | 2/5 |
| $10^{-3}$ | | | | | | | | | | | 0/3 |
| $10^{-4}$ | | | | | | | | | | | 0/4 |
| $10^{-5}$ | | | | | | | | | | | 0/3 |
| $10^{-6}$ | | | | | | | | | 1 | | 1/4 |
| Pooled chorioallantoic membrane | | | | | | | | | | | |
| $10^{-1}$ | | | | 1 | | | | | | | 1/5 |
| $10^{-2}$ | | | | | | | | | | | 0/4 |
| $10^{-3}$ | | | | | | | | 1 | 1 | | 2/5 |
| $10^{-4}$ | | | | | | | | | | | 0/5 |
| $10^{-5}$ | | | | | | | | | 1 | | 1/5 |
| $10^{-6}$ | | | | | | | | 1 | | | 1/5 |
| Pooled allantoic fluid | | | | | | | | | | | |
| $10^{-1}$ | | | | | | | | 1 | | | 1/3 |
| $10^{-2}$ | | | | | | 1 | | | | | 1/4 |
| $10^{-3}$ | 1 | | 1 | | | | | | | | 2/4 |
| $10^{-4}$ | | | | | | | | | | | 0/4 |
| $10^{-5}$ | | | | | | | | | | | 0/3 |
| $10^{-6}$ | | | | | | | | | | | 0/3 |

Inoculation on to the chorioallantoic membrane of 7-day-old embryos resulted in a few embryo deaths, but the mortality was not consistent with the concentrations of the inoculum used. Table 1 represents the type of results obtained.

Inoculation into the allantoic sac produced a greater number of embryo deaths as shown by table 2, but the results were not uniform enough to justify a calculation of the concentration of the agent in the material tested.

Inoculation into the yolk sac caused embryo deaths in relation to the concentration of the inoculum used, both in respect to the number of embryos killed and to the time at which embryo death occurred. This is shown in table 3.

The concentration of the agent in the various portions of the embryo was determined in three pooled samples by the yolk sac route of inoculation. All of the materials tested were infective, with the highest concentrations of viable agent present in the yolk sac membrane and the lowest in the allantoic fluid. These results are summarized in table 4.

*Concentration of the Agent in Allantoic Fluid and Yolk at Intervals Following Inoculation.*—The concentrations of viable agent obtained from embryos killed by the agent suggested that there was probably some period between inoculation and the time of death of the embryo when a maximum concentration of viable agent was present. A series of 70, 7-day-old chicken embryos was inoculated into the yolk sac with a 1 : 10 dilution of infective yolk. At forty-eight, seventy-two, and ninety-six hours after inoculation, random samples were checked for concentration of the agent present in the allantoic fluids and yolk. Only 4 embryos were surviving at ninety-six hours, therefore concluding the experiment. The results tabulated in table 5 demonstrate a maximum concentration in surviving embryos at seventy-two hours after inoculation.

*Effects of Storage Temperature on Viability of Infective Yolk and Allantoic Fluids.*—The effect of storage temperatures on viability of the agent present in the yolk and the allantoic fluid of infected embryos was determined for 37, 30, 10, 0, and —40 C. The materials stored were harvested from 24 embryos within thirty-six hours after death and pooled. The pooled yolk and pooled allantoic fluid were each thoroughly mixed and dispensed into sterile screw-cap vials. Each tube contained 1 ml. of the material tested. Ten tubes each of yolk and of allantoic fluid were then stored at each of the above temperatures. Titration of samples of each held at the various temperatures were made at various intervals. The titration results are shown in tables 6 and 7.

To get additional data on the effects of temperature, particularly at incubator and refrigerator temperatures, a second series of assays was made on infective yolk and allantoic fluids. The yolk and allantoic fluid from 15 infected embryos were harvested and each pooled. Five aliquots of each were placed in sterile screw-cap vials.

**TABLE 2—Deaths of 7-Day-Old Chicken Embryos Between the Third and Twelfth Days After Inoculation into the Allantoic Chamber of 0.2 Milliliters Each of Tenfold Dilutions of Various Portions of Embryos Infected with the Sinusitis Agent**

| Dilution | Day of death 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Final mortality |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Pooled yolk sac membrane** | | | | | | | | | | | |
| $10^{-1}$ | 1 | 1 | | | 1 | | 1 | | | | 4/5 |
| $10^{-2}$ | | 1 | 1 | | | | 1 | | | | 3/3 |
| $10^{-3}$ | | | | | 1 | | | | 1 | | 2/4 |
| $10^{-4}$ | | | 1 | 1 | | 1 | | | | | 3/4 |
| $10^{-5}$ | | | | | | | 1 | | 1 | | 2/5 |
| $10^{-6}$ | | | | | | | | | | 1 | 1/5 |
| **Pooled yolk** | | | | | | | | | | | |
| $10^{-1}$ | | | 1 | 1 | | | | | 1 | | 3/5 |
| $10^{-2}$ | | | 2 | 1 | | | 1 | | 1 | | 5/5 |
| $10^{-3}$ | | | | | 1 | | 1 | | 1 | 1 | 4/5 |
| $10^{-4}$ | | | | 1 | 1 | | 1 | | | | 3/5 |
| $10^{-5}$ | | | | | | | 2 | | 1 | | 3/5 |
| $10^{-6}$ | | | | | | | | | 1 | | 1/5 |
| **Pooled chorioallantoic membrane** | | | | | | | | | | | |
| $10^{-1}$ | | | | 1 | 1 | | | | | | 2/4 |
| $10^{-2}$ | | | | 1 | 1 | | | 2 | 1 | | 5/5 |
| $10^{-3}$ | | | | 2 | | | | | | 1 | 3/3 |
| $10^{-4}$ | | | | | | | | | | | 0/5 |
| $10^{-5}$ | | | | | 1 | | | | | | 1/5 |
| $10^{-6}$ | | | | | | | | | | | 0/3 |
| **Pooled allantoic fluid** | | | | | | | | | | | |
| $10^{-1}$ | | | 2 | 1 | 1 | | | | | 1 | 5/5 |
| $10^{-2}$ | | | | 2 | 1 | | | | | | 3/5 |
| $10^{-3}$ | | | | 1 | | 1 | | | | 1 | 3/4 |
| $10^{-4}$ | | | | 1 | | | | | | | 1/5 |
| $10^{-5}$ | | | | | | | | | | | 0/5 |
| $10^{-6}$ | | | | | | | | | | | 0/5 |

**TABLE 3—Deaths of 7-Day-Old Chicken Embryos Between the Third and Twelfth Days After Inoculation into the Yolk Sac of 0.2 Milliliters Each of Tenfold Dilutions of Various Portions of Chicken Embryos Infected with the Sinusitis Agent**

| Dilution | Day of death 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Final mortality |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Pooled yolk sac membrane** | | | | | | | | | | | |
| $10^{-1}$ | 1 | 2 | 1 | | 1 | | | | | | 5/5 |
| $10^{-2}$ | | 1 | 1 | 1 | | | 2 | | | | 5/5 |
| $10^{-3}$ | 1 | | | 2 | 1 | | 1 | | | | 5/5 |
| $10^{-4}$ | | | | 1 | | | 2 | | | 1 | 4/5 |
| $10^{-5}$ | | | | 1 | | 1 | 1 | | | | 3/5 |
| $10^{-6}$ | | | | | 1 | | | | 2 | 1 | 4/5 |
| **Pooled yolk** | | | | | | | | | | | |
| $10^{-1}$ | | 1 | 2 | | | 1 | | | 1 | | 5/5 |
| $10^{-2}$ | | | 2 | 2 | | 1 | | | | | 5/5 |
| $10^{-3}$ | | | 2 | 3 | | | | | | | 5/5 |
| $10^{-4}$ | | | | | 1 | | 2 | | | 1 | 4/5 |
| $10^{-5}$ | | | | 1 | | | | | 1 | | 2/5 |
| $10^{-6}$ | | | | | | | | | | 1 | 1/5 |
| **Pooled chorioallantoic membrane** | | | | | | | | | | | |
| $10^{-1}$ | 1 | 1 | 1 | 1 | | 1 | | | | | 5/5 |
| $10^{-2}$ | | | 4 | 1 | | | | | | | 5/5 |
| $10^{-3}$ | | 1 | | 1 | | | 1 | | 1 | | 4/4 |
| $10^{-4}$ | | | | | | | | | | 1 | 1/5 |
| $10^{-5}$ | | | | | | | | | 2 | 1 | 3/5 |
| $10^{-6}$ | | | 1 | 1 | | | | | 1 | 1 | 4/5 |
| **Pooled allantoic fluid** | | | | | | | | | | | |
| $10^{-1}$ | | | 2 | 2 | | | 1 | | | | 5/5 |
| $10^{-2}$ | | | 1 | 1 | | | | 1 | 1 | | 4/5 |
| $10^{-3}$ | | | 1 | | | 2 | | | | 1 | 4/5 |
| $10^{-4}$ | | | | | | | | 2 | | | 2/4 |
| $10^{-5}$ | | | | | | | | | | | 0/4 |
| $10^{-6}$ | | | | | | | | | | | 0/5 |

**TABLE 4—Concentration of the Agent of Infectious Sinusitis in Various Portions of the Infected Embryos, Determined by Inoculations into the Yolk Sac and Expressed as $L.D._{50}$ Doses per Milliliter or per Gram for 7-Day-Old Embryos**

| Material tested | Experiment 1 | Experiment 2 | Experiment 3 | Average $l.d._{50}$ doses/ml. or/Gm. |
|---|---|---|---|---|
| Yolk sac membrane | $10^{6.7}$ | $10^{6.2}$ | $10^{6.4}$ | $10^{6.5}$ |
| Yolk | $10^{5.5}$ | $10^{6.7}$ | $10^{5.7}$ | $10^{6.0}$ |
| Chorioallantoic membrane | $10^{6.0}$ | $10^{6.1}$ | $10^{5.5}$ | $10^{5.9}$ |
| Allantoic fluid | $10^{4.5}$ | $10^{4.9}$ | $10^{4.7}$ | $10^{4.7}$ |

**TABLE 5—Concentration of the Agent Present in Allantoic Fluid and Yolk at Intervals Following the Inoculation of Infective Yolk into the Yolk Sac of 7-Day-Old Chicken Embryos Expressed as $L.D._{50}$ Doses per Milliliter for 7-Day-Old Chicken Embryos**

| Time of harvest following inoculation | $l.d._{50}$ doses/ml. of allantoic fluid | $l.d._{50}$ doses/ml. of yolk |
|---|---|---|
| 48 hours (5 living embryos) | $10^{5.2}$ | above $10^{6.7}$ |
| 72 hours (5 dead embryos) | $10^{4.9}$ | $10^{7.3}$ |
| 72 hours (4 living embryos) | $10^{7.0}$ | $10^{9.0}$ |
| 96 hours (5 dead embryos) | $10^{4.9}$ | $10^{7.0}$ |
| 96 hours (4 living embryos) | $10^{6.1}$ | $10^{9.3}$ |

**TABLE 6—The Concentration of the Etiological Agent of Turkey Sinusitis in the Allantoic Fluid of Infected Embryos at Certain Intervals of Storage at Various Temperatures as Determined in $L.D._{50}$ Doses per Milliliter by Inoculation into the Yolk Sac of 7-Day-Old Chicken Embryos**

| Storage time (days) | Storage temperature 37 C. | 30 C. | 20 C. | 10 C. | 0 C. | —40 C. |
|---|---|---|---|---|---|---|
| 0 | $10^{6.9}$ | $10^{6.9}$ | $10^{6.9}$ | $10^{6.9}$ | $10^{6.9}$ | $10^{6.9}$ |
| 1 | $10^{5.2}$ | $10^{6.9}$ | $10^{6.9}$ | $10^{5.2}$ | $10^{5.1}$ | $10^{5.7}$ |
| 2 | $10^{6.0}$ | $10^{6.5}$ | .... | .... | .... | .... |
| 3 | $10^{5.0}$ | $10^{5.5}$ | .... | .... | .... | .... |
| 4 | $10^{1.7}$ | $10^{5.9}$ | .... | .... | .... | .... |
| 5 | 0* | $10^{1.9}$ | $10^{4.7}$ | $10^{5.7}$ | $10^{4.9}$ | $10^{5.9}$ |
| 10 | 0 | $10^{2.5}$ | $10^{2.5}$ | $10^{4.1}$ | $10^{1.7}$ | $10^{4.8}$ |
| 21 | 0 | 0 | 0 | 0 | 0 | $10^{2.5}$ |
| 30 | .... | .... | .... | .... | .... | $10^{2.7}$ |
| 45 | .... | .... | .... | .... | .... | $10^{3.1}$ |
| 60 | .... | .... | .... | .... | .... | $10^{2.4}$ |
| 90 | .... | .... | .... | .... | .... | $10^{2.2}$ |
| 180 | .... | .... | .... | .... | .... | $10^{4.5}$ |

*The designation 0 indicates that no deaths resulted in embryos inoculated into the yolk sac with 0.2 ml. each of a $10^{-1}$ dilution of the material tested.

Three samples of each were stored at 37 C., and two of each were stored in a refrigerator which operated between 6 and 8 C. The results of titrations on these materials held in the incubator are shown by table 8. The results of storage in the refrigerator are recorded in table 9.

**TABLE 7—The Concentration of the Etiological Agent of Turkey Sinusitis in the Yolk of Infected Embryos at Certain Intervals of Storage at Various Temperatures as Determined in $L.D._{50}$ Doses per Milliliter by Inoculation into the Yolk Sac of 7-Day-Old Chicken Embryos**

| Storage time (days) | Storage temperature | | | | | |
|---|---|---|---|---|---|---|
| | 37 C. | 30 C. | 20 C. | 10 C. | 0 C. | —40 C. |
| 0 | $10^{8.1}$ | $10^{8.1}$ | $10^{8.1}$ | $10^{8.1}$ | $10^{8.1}$ | $10^{8.1}$ |
| 1 | $10^{6.7}$ | $10^{6.7}$ | $10^{6.7}$ | $10^{6.7}$ | $10^{6.7}$ | $10^{6.4}$ |
| 2 | $10^{6.5}$ | $10^{6.2}$ | .... | .... | .... | .... |
| 3 | $10^{6.0}$ | $10^{6.0}$ | .... | .... | .... | .... |
| 4 | $10^{5.2}$ | $10^{5.7}$ | .... | .... | .... | .... |
| 5 | $10^{4.7}$ | $10^{5.7}$ | $10^{5.7}$ | $10^{6.5}$ | $10^{5.5}$ | $10^{6.0}$ |
| 10 | 0* | $10^{4.7}$ | $10^{5.2}$ | $10^{5.2}$ | $10^{5.2}$ | $10^{5.0}$ |
| 21 | 0 | 0 | $10^{3.5}$ | $10^{5.0}$ | $10^{5.7}$ | $10^{4.3}$ |
| 30 | .... | .... | $10^{2.0}$ | $10^{4.5}$ | $10^{4.2}$ | $10^{5.2}$ |
| 45 | .... | .... | $10^{2.0}$ below | $10^{3.5}$ below | $10^{2.0}$ | $10^{5.0}$ |
| 60 | .... | .... | $10^{1.7}$ | $10^{1.7}$ below | $10^{1.0}$ below | $10^{5.2}$ |
| 90 | .... | .... | 0 | $10^{1.7}$ | $10^{1.7}$ | $10^{5.1}$ |
| 180 | .... | .... | .... | .... | .... | $10^{6.0}$ |

*The designation 0 indicates that no deaths resulted in embryos inoculated into the yolk sac with 0.2 ml. each of a $10^{-1}$ dilution of the material tested.

## DISCUSSION

The results obtained by using various routes of inoculation indicate that the agent could not be consistently established on the chorioallantoic membranes to produce embryo mortality. Better results were obtained by inoculating into either the allantoic or the yolk chamber. The latter method was the most effective route and was selected as the one of choice. This confirms the work reported by Groupé and Winn.[5]

All portions of the infected embryo that were tested contained appreciable concentrations of the infective agent. Both yolk sac membranes and yolk showed the highest concentrations of viable agents. Because of the ease of handling yolk and the compatibility of this material with the yolk sac route of inoculation, it was selected as the chief source of the agent for further study.

Random samples from infected embryos at various intervals following inoculation demonstrated that the living embryos contained more of the viable agent than did the dead embryos. It was also apparent that the concentrations in the yolk were always higher than in the allantoic fluid. The concentration of the agent in infected chicken embryos was highest in living embryos after a lapse of seventy-two to ninety-six hours following inoculation.

The susceptibility of chicken embryos inoculated at six, seven, eight, nine, and ten days of incubation showed considerable variation. Death of the embryo could not be used as a criterion of infection in embryos inoculated at nine or ten days of incubation. Those inoculated at an earlier age showed characteristic infection patterns as measured by embryo death. There was little variation shown in $l.d._{50}$ values obtained by infecting 6- and 7-day-old chicken embryos. The $l.d._{50}$ dose for the 8-day-old embryo was 10 to 100 times that for the 7-day-old embryo. Because less inoculation injury and mortality was experienced when 7-day-old embryos were used, they were selected for most of the work.

The effects of temperature on viability of the infectious agent of turkey sinusitis indicate that it is more resistant to stor-

**TABLE 8—A Comparison of the Decline in Concentration of the Etiological Agent of Turkey Sinusitis in Allantoic Fluid and Yolk of Infected Chicken Embryos During Storage at 37 C.***

| Storage time (days) | Allantoic fluid stored at 37 C. | | | Yolk stored at 37 C. | | |
|---|---|---|---|---|---|---|
| | Sample A | Sample B | Sample C | Sample A | Sample B | Sample C |
| 0 | $10^{6.2}$ | $10^{6.2}$ | $10^{6.2}$ | $10^{7.2}$ | $10^{7.2}$ | $10^{7.2}$ |
| 1 | $10^{6.2}$ | .... | .... | $10^{7.2}$ | .... | .... |
| 3 | $10^{5.2}$ | .... | .... | $10^{6.7}$ | .... | .... |
| 4 | $10^{3.7}$ | $10^{3.7}$ | $10^{2.7}$ | .... | .... | .... |
| 6 | $10^{1.7}$ | $10^{1.7}$ | 0 | $10^{4.2}$ | $10^{1.7}$ | $10^{3.1}$ |
| 7 | $10^{0.7}$ | $10^{0.7}$ | .... | $10^{4.0}$ | $10^{3.4}$ | $10^{3.7}$ |
| 9 | .... | .... | .... | .... | $10^{4.0}$ | $10^{3.9}$ |
| 11 | .... | .... | .... | .... | $10^{2.0}$ | $10^{2.2}$ |

*Concentrations are expressed as $l.d._{50}$ doses per milliliter for 7-day-old chicken embryos inoculated via the yolk sac.

age in yolk than it is in allantoic fluid. At 37 C., the viability of infected allantoic fluid was destroyed in five to seven days. At temperatures ranging from 0 to 30 C.,

TABLE 9—A Comparison of the Decline in Concentration of the Etiological Agent of Turkey Sinusitis in Allantoic Fluid and Yolk of Infected Chicken Embryos During Storage at 6 to 8 C.*

| Storage time | Allantoic fluid stored at 6 to 8 C. | | Yolk stored at at 6 to 8 C. | |
|---|---|---|---|---|
| (days) | Sample A | Sample B | Sample A | Sample B |
| 0 | $10^{6.2}$ | $10^{6.2}$ | $10^{7.2}$ | $10^{7.2}$ |
| 1 | $10^{5.2}$ | .... | $10^{5.5}$ | .... |
| 3 | $10^{4.2}$ | .... | $10^{6.5}$ | .... |
| 7 | $10^{5.2}$ | $10^{5.2}$ | $10^{5.2}$ | $10^{5.7}$ |
| 9 | $10^{4.7}$ | $10^{4.7}$ | $10^{5.2}$ | $10^{5.7}$ |
| 11 | 0 | 0 | .... | .... |
| 15 | .... | .... | $10^{4.2}$ | $10^{5.5}$ |

*Concentrations are expressed as $l.d._{50}$ doses per milliliter for 7-day-old chicken embryos inoculated via the yolk sac.

viability was lost between ten and twenty days of storage. Allantoic fluid stored at —40 C. remained viable for 180 days. Infected yolk stored at 37 C. lost its activity sometime between five and ten days of storage. At 30 C., it was not infective after twenty-one days of storage and, at 20 C., it was inactive after sixty days of storage. When yolk was stored at 10 and 0 C., it remained viable for at least ninety days and, when stored at —40 C., it was active after 180 days.

The storage of infective yolk caused a uniform loss in viability of the agent during the first twenty-four hours. Subsequent to this period, the materials stored at different temperatures showed a uniform drop in infectivity which appeared to be related to the particular storage temperature. The data indicate that storage of the agent in yolk at —40 C. caused a marked initial fall in the $l.d._{50}$ titer, followed by a slight but uniform drop in titer as the storage time increased. After 180 days storage at —40 C., the yolk was still highly infective, containing $10^{6.0}$ $l.d._{50}$ doses of viable agent per milliliter.

## CONCLUSIONS

1) The causative agent of infectious sinusitis of the turkey will cause death of the chicken embryo when it is inoculated into the yolk sac at six or seven days of incubation.

2) Chicken embryos infected with this agent develop an appreciable concentration of it in the yolk sac membrane, yolk, chorioallantoic membranes, and the allantoic fluid.

3) After 15 or more serial passages of this agent, subcutaneous hemorrhages are prominent in infected embryos.

4) Concentrations of the agent as high as $10^{9.0}$ lethal $dose_{50}$ doses per milliliter are obtained in the yolk of infected embryos when harvested seventy-two hours after inoculation.

5) The agent survives longer in yolk than it does in allantoic fluid, and will survive in the yolk from infected embryos at least 180 days when stored at —40 C.

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# A Quantitative Study of Newcastle Disease Virus in Tissues of Infected Chickens

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FEW QUANTITATIVE studies of Newcastle disease virus (NDV) in the tissues of the chicken have been done. Karzon and Bang have reported in an abstract[1] work in which the concentration of virus was followed in the blood and viscera after inoculation of two different strains of virus by the intramuscular and intracerebral routes. The antibody production was also determined.

Miller and Miller[2] have reported on the extent to which NDV remains in the blood and tracheal mucus of birds following intratracheal and intramuscular inoculation.

For a review of the distribution of NDV in the infected chicken, the reader is referred to the original[3] and the addendum[4] review of the literature on Newcastle disease.

This investigation was carried out in order to study the concentration of NDV virus in tissues of chickens experimentally inoculated with a typical field strain, to compare the virus concentration in tissues following inoculation of three other strains considered to be less virulent, and to follow the antibody production with the four strains studied.

## MATERIALS AND METHODS

All chickens, except one group of cockerels, used in the experimental work were White Leghorn pullets from 9 to 12 weeks of age. The strains of virus studied were identified as: 228-Iowa-1948, a field strain in an early embryo passage; 146-Nebraska-1948, a strain isolated at the Veterinary Research Institute in July, 1948, from contaminated fowlpox vaccine; Roakin-New Jersey-1946, a commercial vaccine strain obtained from Vineland Laboratory live virus vaccine—serial No. 626; and B1—the Hitchner strain of virus obtained through the courtesy of Dr. S. B. Hitchner. The last three listed were considered to be of low virulence for chickens. The number or name used in the designation of each strain is that used at the repository for NDV strains at the University of Wisconsin, Madison. Each inoculating dose of virus was used in the form of allantoic fluid from infected embryos diluted 1 : 10 in broth. The dose employed was 0.1 ml. of the 1 : 10 dilution.

The procedure followed was to inoculate a group of chickens and to remove 2 birds, at 24-hour intervals, from which blood and tissues were collected. The virus concentration in the blood was determined within forty-eight hours after collection by inoculation of embryos with a series of fivefold dilutions. Tissues were frozen at —35 C. until they could be titrated. At that time, the tissue was removed from the freezer, weighed, ground with alundum, and diluted with broth to make a 1 : 5 dilution. The suspension was centrifuged at approximately 1,500 revolutions per minute for five minutes, and the supernatant fluid was used for making tenfold dilutions for embryo inoculations. As a rule, 4 embryos were used for each dilution. The titer was determined by the method of Reed and Muench.[5] Newcastle disease virus infection in the embryo was based upon the rapid hemagglutination test, embryo lesions, and the embryo mortality pattern.

The neutralizing capacity of the serum was tested according to standard procedure.[6] The hemagglutination-inhibition (HI) test was done according to the method by Salk,[7] except that the serum dilutions were made directly in the virus suspension and the amount of virus used was 20 hemagglutinating units when calculated on the basis of final dilutions. The results of the HI test were expressed as HI units. An HI unit is the amount of serum antibody which will neutralize one hemagglutination unit in the test. The strain of NDV used in the tests for antibodies was 174-Iowa-1947.

With the exception of groups Roakin intranasal and 228 intranasal, each inoculation was repeated at a later time, so that the results from 4 birds rather than from 2 could be obtained for each tissue.

## RESULTS

To study the concentration of NDV in the tissues of chickens experimentally inoculated with a typical field strain, birds

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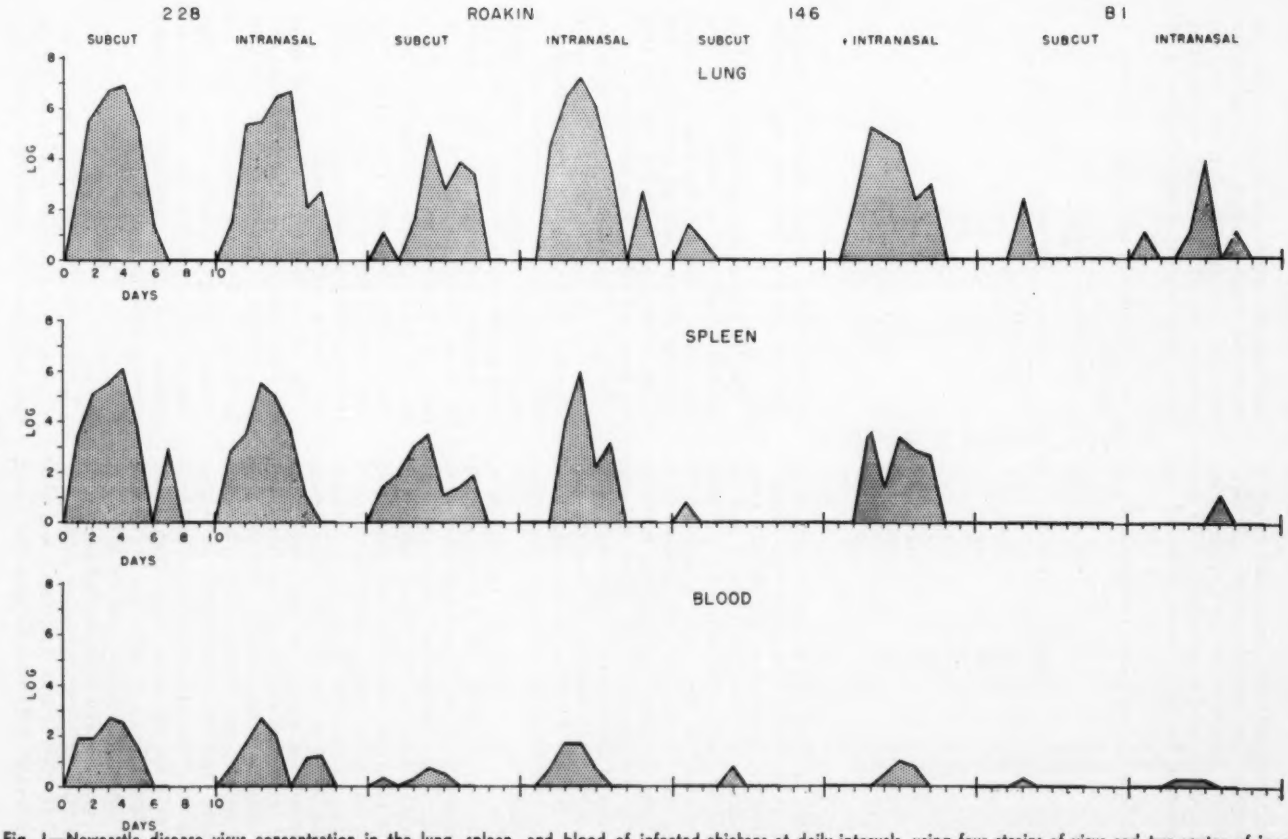


Fig. 1—Newcastle disease virus concentration in the lung, spleen, and blood of infected chickens at daily intervals, using four strains of virus and two routes of inoculation. Expressed as the logarithm of the average number of embryo lethal doses per 1 ml. of tissue suspension.

were inoculated with strain 228 either intranasally or subcutaneously. Table 1 gives the logarithm of the average embryo lethal doses (e.l.d.'s) per milliliter of tissue suspension for each tissue, following inoculation of virus 228 subcutaneously in the web of the wing. The virus was found to be present in the blood and tissues, except the brain, at twenty-four hours and persisted in some of the tissues for 168 hours, after which no virus could be isolated. The lung and trachea gave the highest yields of virus. In table 2 is listed the logarithm of the average for each tissue following intranasal inoculation with strain 228. This group was not repeated so that the averages for only 2 birds could be used. The results, in general, are similar to those shown in table 1.

TABLE 1—Concentration of Newcastle Disease Virus in Tissues Following Subcutaneous Inoculation with Strain 228

| Hours after inoc. | Log of average e.l.d.'s per ml. of tissue suspension | | | | | |
|---|---|---|---|---|---|---|
| | Blood | Spleen | Brain | Lung | Trachea | Intestinal contents |
| 24 | 1.88 | 3.44 | 0.00 | 3.09 | 2.91 | 0.78 |
| 48 | 1.88 | 5.14 | 1.40 | 5.45 | 7.12 | 2.24 |
| 72 | 2.68 | 5.53 | 1.34 | 6.66 | 7.03 | 3.90 |
| 96 | 2.46 | 6.07 | 4.91 | 7.03 | 7.66 | 2.52 |
| 120 | 1.52 | 3.73 | 3.20 | 5.18 | 6.46 | 2.44 |
| 144 | 0.00 | 0.00 | 1.11 | 1.28 | 3.90 | 0.78 |
| 168 | 0.00 | 2.89 | 0.00 | 0.00 | 0.78 | 0.00 |
| 192 | ... | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 216 | ... | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 240 | ... | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

The results following inoculation of virus strain 228 served as a standard for comparison in the second objective of the study. The comparative results of virus concentrations in the blood, spleen, and lung following inoculation of virus strain 228 and three other strains considered to be less virulent for chickens are presented in fig. 1. There was considerable difference between the results of the subcutaneous and intranasal routes of inoculation with the Roakin strain of NDV. The virus was not isolated consistently from the various tissues following subcutaneous inoculation. This difference is more apparent in the results from individual birds than in the averages. The concentration of virus in the tissues was greater and the titers were less variable following intranasal inoculation than after subcutaneous inoculation.

With NDV strain 146, there was again considerable difference between the results following the two routes of inoculation. The intranasal route resulted in a generalized infection, although the virus was not consistently found in high concentration. After subcutaneous inoculation, the virus was only found occasionally in the tissues, although antibody response was satisfactory.

The B1 strain was apparently the least virulent of the strains studied. The virus was seldom isolated in the tissues sampled following either route of inoculation, but a rise in antibody indicated that the bird had become infected with the virus.

In fig. 2, the average neutralizing capacity of the serum is given for each group following intranasal and subcutaneous inoculations. Tests for antibodies were not made after the eighth day following intranasal inoculation with strain 228. As might be expected, the field strain 228 produced the most consistent antibody response. As early as ninety-six hours after subcutaneous inoculation, some neutralizing antibodies were detected. The other three virus strains produced a definite antibody response, but there was considerable variation among individual birds.

The HI antibodies were detected about the same time as the infectivity-neutralizing antibodies. The field strain 228 produced the earliest and most consistent and uniform antibody response, while the other strains stimulated a lesser and more irregular response, particularly following subcutaneous inoculation.

## DISCUSSION

In order to consolidate the results and to make the material more readable, the data are presented as averages rather than

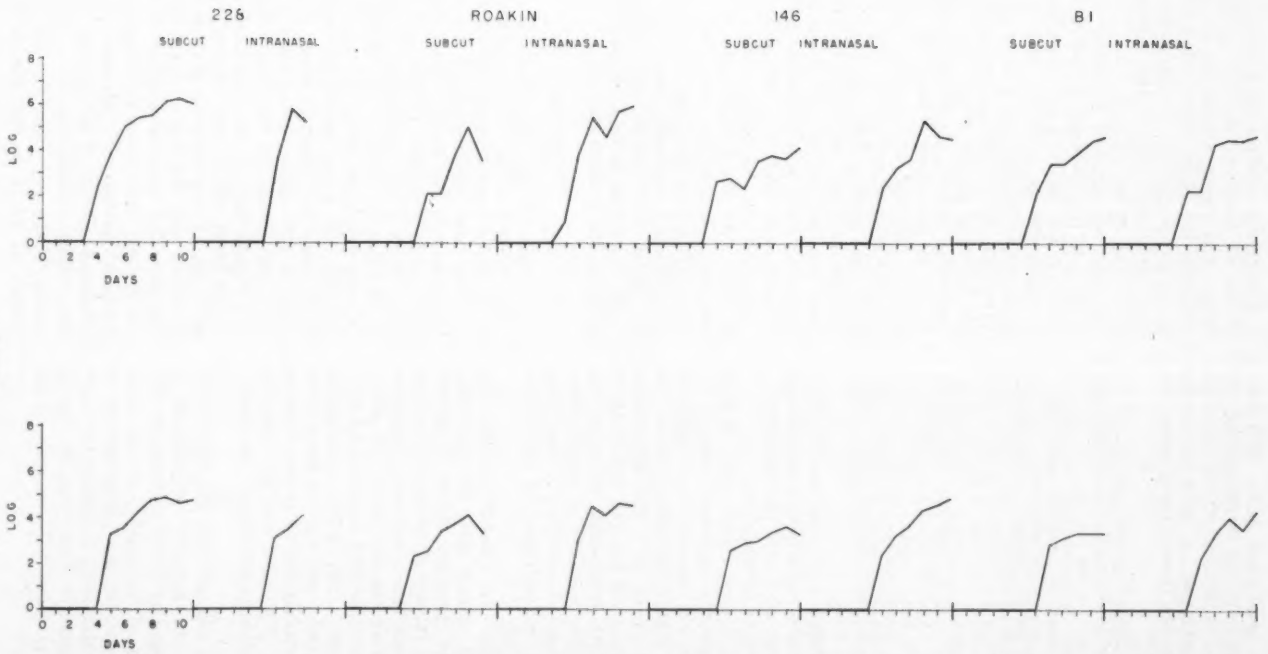


Fig. 2—Neutralizing capacity of serum from Newcastle disease-infected chickens at daily intervals, using four strains of virus and two routes of inoculation. Above: neutralization of virus infectivity expressed as the logarithm of the average number of neutralizing doses. Below: hemagglutination inhibition expressed as the logarithm of the average number of hemagglutination-inhibition units.

results from individual birds. The averages tend to cover up irregularities that were present in results of the titrations following inoculation of the less virulent strains, since the lower titers were frequently outweighed by the higher titers. The averages are the arithmetical averages of the number of e.l.d.'s per milliliter of tissue suspension for each bird.

TABLE 2—Concentration of Newcastle Disease Virus in Tissues Following Intranasal Inoculation with Strain 228

| Hours after inoc. | Log of average e. l. d.'s per ml. of tissue suspension | | | | |
|---|---|---|---|---|---|
| | Blood | Spleen | Brain | Lung | Trachea |
| 24 | 0.78 | 2.80 | 0.00 | 1.54 | 1.11 |
| 48 | 1.72 | 3.46 | 0.00 | 5.44 | 5.05 |
| 72 | 2.71 | 5.55 | 1.64 | 5.51 | 6.46 |
| 96 | 2.00 | 4.96 | 2.83 | 6.49 | 6.50 |
| 120 | 0.00 | 3.70 | 1.74 | 6.74 | 6.90 |
| 144 | 1.08 | 1.11 | 3.89 | 2.07 | 3.39 |
| 168 | 1.18 | 0.00 | 0.00 | 2.66 | 1.40 |
| 192 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

Newcastle disease virus was isolated from experimentally infected birds on the first through the seventh day following exposure by either the intranasal or the subcutaneous route to a virulent strain. In only one instance was the virus isolated after the 168th hour—that being on the eighth day. The inability to isolate virus from the tissues occurred at a time when the antibody concentration in the serum was fairly high.

The averages (fig. 1 and 2) indicate that NDV may be isolated after the antibodies are present at diagnostic levels. While this could be the fallacy of averages, this actually happened in a number of individual cases. Following the inoculation of strain 228, tests for infectivity-neutralizing antibodies indicated over 1,000 neutralizing doses, yet virus was still isolated from some of the tissues. No virus was detected in the blood. This also was the case following intranasal inoculation of the Roakin strain.

The results indicate that there is a wide variation in the response of chickens to different strains of NDV and also to different routes of inoculation of the same strain of virus. If the concentration of virus in the tissues is a measure of virulence for a strain of NDV, then the results can be used to judge the virulence of the strains studied.

The results confirm the avirulent nature of the B1 strain as reported by Hitchner.[8] While this strain stimulated an antibody response, the virus was isolated from only a few tissue samples. It also failed to produce cloudy air sacs in the chickens even after intranasal inoculation. The strain was also less virulent for embryos, as indicated by the delayed mortality pattern.

Strain 146 was also found to have a low virulence for the chickens used in the experiment, although not to the extent of strain B1. Its avirulence was more apparent following the subcutaneous route of inoculation. After intranasal inoculation, cloudy air sacs were frequently observed; while, following subcutaneous inoculation, cloudy air sacs were observed only in a few birds. Strain 146 was also less virulent for embryos, as shown by the longer time required to kill and the stunted appearance of the embryos when killing was delayed until the fifth or sixth day.

Judging by the concentration of virus in the tissues, the Roakin strain, after subcutaneous inoculation, was less virulent than strain 228, but, after intranasal inoculation, its virulence was only slightly less than NDV strain 228. The Roakin strain appeared to be as virulent for embryos as the field strain 228.

## SUMMARY

A study of the concentration of Newcastle disease virus (NDV) in the tissues of chickens experimentally infected with a virulent field strain revealed the virus to be present in the blood, spleen, lung, trachea, and intestinal contents at twenty-four hours after inoculation. It continued to be found in some of the tissues on the seventh day, after which no virus could be isolated. The virus was most concentrated in the lung and trachea, where concentrations of $10^7$ embryo lethal doses per milliliter were found on the fourth day.

A comparison of the virus concentration in the blood, spleen, and lung following inoculation of four different strains of NDV indicated that the less virulent the strain, the lower was the concentration of virus in the tissues. There were more apparent differences between the intranasal and subcutaneous routes of inoculation with the less virulent strains than with the virulent strain. There was usually a lower concentration of virus in the tissues following

subcutaneous inoculation of the less virulent strains.

While antibody production was satisfactory following inoculation of the four strains studied, the virulent field strain produced the most consistently high antibody response. Following inoculation of the less virulent strains, the antibody response showed more variation and was frequently less than the response following inoculation of the more virulent strain. The infectivity-neutralizing antibody and the hemagglutination-inhibition antibody were detected by the fifth to the seventh day in the experimental chickens.

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# Pathogenicity of Salmonella Typhimurium Administered Subcutaneously to Mice in Mixture with Each Polynucleotide Complex from Salmonella Typhimurium, Salmonella Typhosa, and Salmonella Newport

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SALMONELLA TYPHIMURIUM has been found to cause extensive outbreaks of infection among animals and man,[1] and it is a pathogenic agent highly invasive for mice.[2] According to Weil,[2] certain of the Salmonella types possess properties of virulence by means of which the bacteria can penetrate intestinal walls and migrate through lymphatics. *Salmonella typhimurium* has an inherent ability to invade tissues and kill mice. Pike and Mackenzie[3] and Mackenzie, Pike, and Swinney[4] have expressed the opinion that polysaccharides of *S. typhimurium* are not determinants of virulence of this species in mice and that unknown chemical determinants which are present in the bacteria are serologically inactive. Mackenzie and Brewster[5] have observed that strains of *S. typhimurium* differing in degrees of virulence contain determinants for high virulence or avirulence for mice. No information is available in the literature of chemical factors in *S. typhimurium* which may be associated with invasiveness of this species.

In this work, an attempt has been made to enhance invasiveness of *S. typhimurium* culture when saline suspensions of the organisms are introduced subcutaneously to mice in mixture with each of highly polymerized polynucleotide complexes from *S. typhimurium*, *Salmonella typhosa*, and *Salmonella newport*. *Salmonella typhosa* possesses a somatic antigen XII in common with *S. typhimurium;* whereas, in the case of *S. newport*, there is no somatic antigenic relationship to mouse typhoid bacteria. Relative concentrations of "purified" polynucleotide complex of each of *S. typhimurium*, *S. typhosa*, and *S. newport* are indicated by the phosphorus content of final preparations. Although the concentrations of phosphorus are not identical, the polynucleotide complexes of these three Salmonella types are prepared by identical chemical procedures.[6]

## MATERIALS AND METHODS

*Cultures.—Salmonella typhimurium*, the causative organism of mouse typhoid, was a smooth, diphasic culture which was isolated from an infected guinea pig at the Seventh Service Command Medical Laboratory, Fort Omaha, Omaha, Neb., in 1945. *Salmonella newport*, culture 704, was an R (rough) type strain from the Dr. E. O. Jordan stock culture collection, University of Chicago. *Salmonella typhosa*, identified as T290, was obtained from the same source.

*Viable Cell Counts.*—The number of organisms of broth cultures of *S. typhimurium* was estimated by plate colony count method. In each of two experiments described, approximately 6½-hour-old cultures were diluted tenfold, serially, in sterile 0.85 per cent sodium chloride solution. The diluted suspensions of bacteria were left standing at room temperature during the experimental period. Veal-infusion agar medium was employed for colony count determinations, indicating the maximum number of viable cells at the end of the experimental interval. Two cultures employed in this study contained 11 x $10^7$ and 14 x $10^8$ viable bacteria per milliliter of broth culture, respectively.

*Purified Polynucleotide Complex.*—The au-

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The research report was undertaken in coöperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces, under the supervision of Dr. Gail M. Dack of the Food Research Institute, University of Chicago, and has been assigned No. 328 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

The author is indebted to Dr. Gail M. Dack, director of the Food Research Institute, University of Chicago, for his advice and criticism of the manuscript.

At present, the author is in the Veterinary Division, Army Medical Service Graduate School, Washington 12, D. C.

thor[6] has described a chemical method for isolating, refining, and concentrating polynucleotide substances from rough and smooth cultures of several Salmonella types, namely, *S. typhosa*, *Salmonella enteritidis*, *Salmonella montevideo*, *S. newport*, *Salmonella pullorum*, and *S. typhimurium*. Briefly, the following procedure was used: A solution of 0.1 molar sodium citrate and 0.2 per cent sodium desoxycholate, pH 7.3, was employed to disrupt masses of gram-negative bacilli. Utilization was made of differences in solubilities of nucleoproteins and nucleic acids in solutions of sodium citrate-sodium desoxycholate and molar sodium chloride, and in 95 per cent ethyl alcohol. A highly polymerized bacterial nucleoprotein was liberated from the disrupted gram-negative organisms and was then precipitated with ethyl alcohol in the form of a sticky fibrous material. The bacterial nucleoprotein was redissolved in the citrate desoxycholate and molar sodium chloride solutions and was deproteinized five times with one-half the volume of a modified Sevag mixture of one part iso-amyl alcohol and four parts of chloroform. The above described, relatively mild chemical-purification steps resulted in the isolation from Salmonella organisms of a fibrous material which was dissolved in molar sodium chloride solution and which has been designated purified polynucleotide complex.

The organic compound from *S. typhosa* was studied extensively. The general chemical composition (nitrogen : phosphorus ratios of 1.60 and 1.61), colorimetric reactions for pentose[7] and desoxypentose[8] carbohydrates, and ultraviolet light-absorption spectra, as measured with a Beckman quartz spectrophotometer, indicated that polymerized substances which were isolated from *S. typhosa* contained, primarily, desoxypentosenucleic acid contaminated with relatively small amounts of ribonucleic acid. The bacterial nucleic acid complexes, from each of three Salmonella types employed in these experiments, were prepared by identical chemical procedures and their concentrations indicated by their phosphorus content as follows: *S. typhimurium*, 255 $\mu g.$ of phosphorus per milliliter; *S. newport*, 220 $\mu g.$ of phosphorus per milliliter; and *S. typhosa*, 156 $\mu g.$ of phosphorus per milliliter of final preparation.

*Sterilization of Purified Polynucleotide Complex.*—Twenty-five-milliliter aliquots of the polynucleotide preparations were blown from a pipette into three volumes of 95 per cent ethyl alcohol and the mixtures stored at 4 to 6 C. for twenty-four to forty-eight hours. A fibrous precipitate appeared immediately after ejecting the nucleic acid mixture into the alcohol. Following the period of refrigeration, the alcohol was decanted and the fibrous material drained for several seconds and then dissolved in 25 ml. of sterile physiological saline solution.

*Experimental Mice.*—White mice, weighing 12 to 15 Gm., were obtained from one source. Upon arrival, they were vigorous and showed no evidence of intestinal infection. Mice were inoculated with test materials approximately one day after arrival at the laboratory. Survivors were counted for eight days. At the end of each experiment, control mice inoculated with materials other than those containing Salmonella organisms were killed and not found to harbor Salmonella. Test animals dead from infection were selected at random and examined postmortem. Pure cultures of Salmonella organisms were isolated from the heart and several other organs of these mice.

*Test Cell Suspensions.*—Diluted cell suspensions of *S. typhimurium*, in 0.25- and 0.10-ml. volumes, were mixed with 1-ml. volumes of saline solution and saline solution containing dissolved bacterial polynucleotide complex. The test bacterial suspensions were prepared during a period of about thirty minutes and were then stored at 4 to 6 C. until about thirty minutes prior to injection into experimental mice.

*Route of Inoculation.*—Mice were injected subcutaneously in the right flank with each of the test suspensions of mouse typhoid organisms. Doses of 0.25 ml. were injected into mice with a 25-gauge needle attached to a tuberculin-type syringe.

*Duration of Experimental Infection.*—Experimental infection in mice was arbitrarily limited to eight days. Survivors were counted twice daily.

*Measurement of Virulence.*—The lethal dose$_{50}$ (l.d.$_{50}$) was calculated by the method of Reed and Muench.[9] The l.d.$_{50}$ expressed the number of viable organisms that killed 50 per cent of mice injected with *S. typhimurium* organisms during an eight-day experimental period. Fold increase in pathogenicity was an index number of the ratio:

$$\frac{\text{l.d.}_{50} \text{ of cell-saline control}}{\text{l.d.}_{50} \text{ of PPC*-treated cell mixture on the eighth day of infection.}}$$

## RESULTS

Data for two experiments are summarized in table 1. Polynucleotide preparations from *S. typhimurium* (128 $\mu g.$ of phosphorus per 0.5 ml. inoculated per test animal were estimated to be equivalent to 5.8 mg. of PPC substance),* *S. newport* (110 $\mu g.$ of phosphorus per 0.5 ml. = 5.0 mg. of PPC), and *S. typhosa* (78 $\mu g.$ of phosphorus per 0.5 ml. = 3.5 mg. of PPC) were nontoxic

*The reference standard is a preparation of purified polynucleotide complex (PPC) from *S. typhosa*, which contains 2.2 per cent phosphorus by weight.

for mice injected with these bacterial preparations.

Saline suspensions of test *S. typhimurium* culture were relatively avirulent following injection into mice during an eight-day experimental infection (table 1, experimental mouse groups 1 and 4).

Purified bacterial polynucleotide substance from homologous and heterologous Salmonella types repeatedly increased susceptibility of mice to subcutaneous injections with sublethal doses of *S. typhimurium* organisms (table 1, experimental mouse groups 2, 3, 5, 6, and 7). Although purified polynucleotide complex from each of *S. newport* and *S. typhosa* affected fold increases in the pathogenicity of *S. typhimurium* bacilli injected subcutaneously in mice, the virulence-enhancement property of the homologous PPC-mouse typhoid mixture appeared to be more marked.

The results were interpreted to indicate that bacterial polynucleotide complexes, whether prepared from homologous or heterologous Salmonella types, in this case from *S. typhimurium, S. typhosa,* and *S. newport,* possessed a property for enhancing invasiveness of *S. typhimurium* organisms injected subcutaneously into mice in mixture with purified polynucleotide complex.

## DISCUSSION

Invasiveness of microörganisms has been directly or indirectly associated with highly polymerized polysaccharides, such as are found in Pneumococcus organisms and Friedlander's bacillus,[10] with a polypeptide found in the anthrax bacillus and composed of d(-)glutamic acid units,[11] with an M protein and hyaluronidases in group A streptococci,[12, 13] and with a toxic O antigen complex in gram-negative organisms.[14] Felix and Pitt[15] expressed the opinion that the maximum virulence of *S. typhosa* was due to the combined activity of two antigens, namely, the O and Vi antigens. Almon[16] suggested that organisms containing Vi antigen multiplied faster, since the Vi substance provided a protective coating against phagocytosis, and this accounted for the enhanced virulence of typhoid organisms for mice. With the exception of the toxicity of O antigens of gram-negative organisms, the substances cited above are nontoxic to animals and are able in some unknown manner to protect a pathogen against a host's defensive mechanisms.

Purified polynucleotide complex from each of *S. typhosa, S. typhimurium,* and *S. newport* are nontoxic for mice when administered subcutaneously and intraperitoneally (from unreported data) in doses roughly estimated as 3.5, 5.8, and 5.0 mg. of each of the three cultures, respectively. In contrast, Raistrick and Topley -and Boivin-type antigens of *S. typhimurium* are toxic for mice when injected intraperitoneally in doses of about 0.5 to 1 mg.[4]

In this study, it was found that a bacterial substance, purified polynucleotide complex, isolated from *S. typhimurium* cells enhanced the invasiveness of the homologous mouse typhoid culture when injected subcutaneously into mice. Purified polynucleotide complex preparations from *S. typhosa* and R-type *S. newport* cultures modified mouse typhoid infections in mice but not necessarily to the extent of the homologous bacterial PPC. These findings lent support to the work of Mackenzie and his associates who expressed the opinion that cultures of *S. typhimurium* contained nonantigenic determinants for virulence of mouse typhoid organisms in mice. It is generally known that pure bacterial desoxypentosenucleic acids are nonantigenic,[17] and it may be that one probable factor relating to invasiveness of mouse typhoid organisms injected subcutaneously into mice in mixture with each of the polynucleotide complex from *S. typhimurium, S. typhosa,* and *S. newport* may be a nucleic acid moiety associated with PPC of these three Salmonella types.

The mechanism of virulence enhancement of *S. typhimurium* infections in mice by means of homologous and heterologous bacterial polynucleotide complexes is not understood. In attempting to find possible explanations, several modes of action of Salmonella polynucleotide complex may be considered.

1) A wide number of substances of nonbacterial origin have been used to enhance the pathogenicity of intra-abdominally injected microörganisms in mice, and hog gastric mucin has been studied extensively.[18, 19] It is known, for example, that 50 organisms of *S. typhimurium* suspended in mucin prove lethal for 10/10 mice inoculated intraperitoneally.[20] The marked effectiveness of 5 per cent mucin for reducing the $l.d_{50}$ of naturally invasive mouse typhoid organisms has been observed in the author's

laboratory when mucin-cell mixtures are injected subcutaneously into mice.[21] It is suggested that bacterial polynucleotides from the three Salmonella types herein reported may enhance the pathogenicity of *S. typhimurium* culture for mice in a nonspecific manner, as do substances of nonbacterial origin.

2) In contrast to substances of nonbacterial origin, complete polysaccharide antigens of gram-negative organisms have interfered with the bactericidal power of normal and immune serums on Salmonella organisms.[10, 22] It should be noted that McLeod[23, 24] reported that mucin inhibited the bactericidal action of peritoneal fluid of the mouse on Meningococcus organisms *in vitro* and in mice injected intra-abdominally with mucin-Meningococcus mixtures. It may be that components are associated with Salmonella polynucleotide complexes which can inhibit bactericidal action of mouse body fluids when mice are injected subcutaneously with mixtures of mouse typhoid organisms and Salmonella polynucleotide substances.

3) Caspersson[25] has proposed a theory

**TABLE I—Association of Pathogenicity of Salmonella Typhimurium for Mice with Bacterial Polynucleotide Complexes from Homologous Salmonella Typhimurium and Heterologous Salmonella Typhosa and Salmonella Newport Cultures**

| Experimental group and No. mice injected | Diluent | Experiment 1 — No. of *S. typhimurium* organisms injected — 5.5 X | | | | | | | | | | l.d$_{50}$* | Fold increase in pathogenicity** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $10^8$ | $10^5$ | $10^4$ | $10^3$ | $10^2$ | 10 | 1 | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | | |
| 1-32<br>2-38 | Saline | 2/2† | 0/2 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | ... | $3.025 \times 10^6$ | 1.0 |
| | *S. typhimurium* PPC (51 μg. P)‡ | 3/3 | 3/3 | 3/4 | 2/4 | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | 0/4 | $5.5 \times 10^8$ | 550.0 |
| 3-33 | *S. newport* PPC (44 μg. P) | 1/2 | 1/2 | 1/4 | 1/4 | 0/3 | 0/4 | 0/4 | 0/4 | 0/4 | 0/2 | $3.925 \times 10^5$ | 7.7 |

Controls: 0.5 ml. of diluent w/o bacteria: PPC of *S. typhimurium* (128μg. P), 0/2; PPC of *S. newport* (110 μg. P), 0/2.

All injections were made subcutaneously in mice, in the right flank. Volume injected per mouse is 0.25 ml. of diluent and 0.05 ml. of cell suspension of numbers of bacteria indicated. *l.d$_{50}$ is calculated by the method of Reed and Muench for the eighth day after inoculation. **Fold increase in pathogenicity is an index number of the ratio:

$$\frac{\text{l.d}_{50}\text{ of cell-saline control, on the eighth day of infection}}{\text{l.d}_{50}\text{ of PPC-treated cell mixture.}}$$

†Numerator = number of mice dead; denominator = number of mice injected. ‡PPC = "purified" polynucleotide complex from the Salmonella type indicated.

| Experimental group and No. mice injected | Diluent | Experiment 2 — No. of *S. typhimurium* organisms injected — 3.5 X | | | | | | | l.d$_{50}$* | Fold increase in pathogenicity** |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $10^8$ | $10^7$ | $10^6$ | $10^5$ | $10^4$ | $10^3$ | $10^2$ | | |
| 4-28<br>5-28 | Saline | 4/4 | 4/4 | 3/4 | 0/4 | 0/4 | 0/4 | 0/4 | $2.44 \times 10^6$ | 1.0 |
| | *S. typhimurium* PPC (64 μg. P) | 4/4 | 4/4 | 2/4 | 4/4 | 3/4 | 1/4 | 0/4 | $2.97 \times 10^4$ | 82.2 |
| 6-27 | *S. newport* PPC (55 μg. P) | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 | 1/4 | 1/3 | $9.9 \times 10^3$ | 270.0 |
| 7-28 | *S. typhosa* PPC (39 μg. P) | 4/4 | 4/4 | 4/4 | 2/4 | 1/4 | 1/4 | 1/4 | $1.51 \times 10^5$ | 16.3 |

Controls: 0.5 ml. of diluent w/o bacteria: PPC of *S. typhimurium* (128 μg. P), 0/2; PPC of *S. newport* (110 μg. P), 0/2; PPC of *S. typhosa* (78 μg. P), 0/2; uninoculated, 0/2.

Volume injected per mouse is 0.25 ml. of diluent and 0.025 ml. of cell suspension of numbers of bacteria indicated. Mice injected with $3.5 \times 10^8$ cells received 0.25 ml. of *S. typhimurium* culture in addition to the diluents indicated.

which relates the synthesis of cellular protoplasm with the metabolism of nucleic acids in living cells. It is suggested that *S. typhimurium* organisms are provided with optimal conditions for growth and invasiveness in a natural host environment when supplemented with relatively large amounts of mixtures of bacterial desoxypentose and ribonucleic acids from homologous and heterologous Salmonella types.

## SUMMARY

1) "Purified" polynucleotide complex preparations from Salmonella types contained desoxypentosenucleic acid contaminated with ribonucleic acid.

2) Purified polynucleotide complex from *Salmonella typhimurium* was found to possess a property contributing to invasiveness of the homologous culture in mouse typhoid infections experimentally induced in mice.

3) Purified polynucleotide complex from antigenically related *Salmonella typhosa* and antigenically unrelated *Salmonella newport*, under the conditions of these experiments, also enhanced the pathogenicity of mouse typhoid organisms injected subcutaneously into mice.

4) Three possible mechanisms of action of virulence enhancement of *S. typhimurium* bacilli injected subcutaneously into mice in mixture with bacterial polynucleotide complex from *S. typhimurium*, *S. typhosa*, and *S. newport* were discussed.

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# Newcastle Disease Virus-Neutralizing Indexes of Normal Chicken Serums

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APPLICATION OF the *in vitro* serum-neutralization (SN) test to the diagnosis of Newcastle disease (ND), use of the test for determining susceptibility of experimental chickens, and its use for studying the antigenicity of ND vaccines have made it desirable to obtain data on the neutralizing values of normal chicken serums. *In vitro* neutralization of Newcastle disease virus (NDV) employing inoculation upon the chorioallantoic membrane of embryonating chicken eggs was demonstrated by Keogh.[1] Burnet[7] used an SN test in which inoculations were made into the amniotic sac. Minard and Jungherr[2] developed an SN test employing inoculation of the serum-virus mixture into the allantoic chamber of embryonated chicken eggs. A similar procedure was employed by Brandly, Moses, Jungherr, and Jones,[3] who also studied the effects of various routes of inoculation upon SN titers.

This report presents SN values derived by us from 175 serums obtained from apparently normal chickens not having any history of prior infection with NDV.

## MATERIALS AND METHODS

Five flocks of chickens were used as the source of serums. Serum samples were obtained from 60, 8-week-old New Hampshire Red chickens in one flock, from 14, 9-week-old New Hampshire Reds in another flock, and from 81 adult hens of ages varying from 1 to 2 years on another farm. Among the adult hens, approximately equal numbers of three breeds were used, Barred Rocks, White Rocks, and New Hampshire Reds. The parent stock of the chickens providing the blood samples had not been vaccinated for ND, and respiratory infections had not been observed in the flocks. Twenty serums were obtained from 10-week-old New Hampshire Red chickens which were hatched from eggs laid approximately eight months following intranasal vaccination of the parent stock with the B1 strain of NDV.

The blood samples were obtained from the wing vein with syringes and permitted to clot at room temperature. The serums were clarified by centrifugation, measured, and preserved by the addition of phenol to a final concentration of 0.5 per cent. The serums were stored at 6 C. in test tubes closed with rubber stoppers. The tests were completed within three months after the serum samples were obtained from the chickens.

The SN testing procedure was essentially the same as that outlined by Brandly *et al.*[3] Equal quantities of undiluted phenolized serum were mixed with tenfold dilutions of amnioallantoic fluid virus estimated to be appropriate for obtaining end points. The serum-virus mixture was incubated one hour at room temperature before inoculation into the allantoic chamber of 4, 10-day-embryonating chicken eggs for each virus dilution, using 0.1 cc. of inoculum. One series of virus dilutions was used for all serums tested on a given day. The inoculum for the virus titration was 0.05 cc. Deaths of embryos occurring during the first twenty-four hours following inoculation were regarded as nonspecific. Embryos dying from the second through the fourth days were employed in calculating the embryo lethal $dose_{50}$ neutralization indexes ($l.d._{50}$ NI) by the method of Reed and Muench.[5] The presence of NDV in the dead embryos was not determined by hemagglutination or embryo inoculation. Two strains of NDV, Ky-90 and Ky-201, were used for the SN tests. Eggs laid by both immune and nonimmune hens were used in conducting the tests.

## RESULTS

The NDV-neutralizing values obtained with the 175 serums are summarized in table 1. There was no neutralization of NDV by 132 (75.42%) of the serums. Lethal $dose_{50}$ neutralization indexes varying from 1.32 to 10.0 were obtained for 42 (24%) of the serums, and only 1 chicken had an $l.d._{50}$ NI greater than 10, its value being 14.79.

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The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

This study was conducted as a segment of Research and Marketing Act Regional Project S-6, Diagnosis and Control of Newcastle Disease of Poultry. The coöperating agencies under this project are the Kentucky, Texas, and Virginia agricultural experiment stations. The research reported in this paper was done solely at the Kentucky station.

## DISCUSSION

Phenolization of serums prior to SN determinations apparently has no effect on the protective titer. Brandly *et al.*[3] reported that the addition of 1.0 per cent phenol did not result in any difference in titer. Similar studies in our own laboratory failed to show consistent or reproducible differences in SN titers of filtered and phenolized serums.

**TABLE I—Neutralization Indexes Resulting from Serum-Neutralization Tests on 175 Serums from Normal Chickens Without History of Newcastle Disease Virus Infection**

| Neutralization index ($l.d._{50}$ NI)* | Frequency of occurrence | Accumulated percentage of occurrence |
|---|---|---|
| 0.00 | 132 | 75.42 |
| 1.32 | 1 | 75.99 |
| 1.74 | 1 | 76.51 |
| 1.78 | 1 | 77.14 |
| 1.86 | 3 | 78.85 |
| 1.91 | 2 | 79.99 |
| 2.14 | 4 | 82.28 |
| 2.82 | 1 | 82.85 |
| 2.88 | 1 | 83.42 |
| 2.95 | 1 | 83.99 |
| 3.16 | 8 | 88.57 |
| 4.17 | 1 | 89.14 |
| 4.37 | 2 | 90.28 |
| 4.57 | 3 | 91.99 |
| 4.67 | 1 | 92.57 |
| 5.89 | 3 | 94.28 |
| 6.76 | 1 | 94.85 |
| 6.91 | 2 | 95.99 |
| 10.00 | 6 | 99.43 |
| 14.79 | 1 | 100.00 |

*Embryo lethal dose$_{50}$ units of Newcastle disease virus neutralized by 0.05 cc. of serum.

Of the serums tested in this study, 99.43 per cent neutralized 10 or fewer embryo $l.d._{50}$ of NDV. The mean value of the $l.d._{50}$ NI for 175 serums was 1.14 and the standard deviation was 2.5. These results conform closely to those obtained by Cunningham,[6] whose data on 100 SN tests indicated that the $l.d._{50}$ NI of 99.70 per cent of normal chicken serums would not be expected to exceed approximately 11 embryo $l.d._{50}$ per 0.05 cc. of serum. Similar results were recorded by Osteen and Anderson,[4] their data indicating that SN titers of less than $10^1$ were obtained on serum from 23 normal, susceptible chickens. Also, they reported that SN titers as high as 100 minimum lethal doses (m.l.d.) of NDV were not encountered in pooled serum samples from flocks with an ND-negative history. Brandly *et al.*[3] determined the SN activity of 46 serums obtained from chickens without known history of contact with NDV and found neutralizing titers varying from zero to 100 m.l.d. of NDV, 91.3 per cent of their serums having titers of 10 or fewer m.l.d. Variance from these results was obtained by Minard and Jungherr[2] on serums from flocks having no known infection with NDV. In an examination of 25 chicken serums from one flock, they found that 68 per cent neutralized more than 100 embryo $l.d._{50}$ of NDV, the titers ranging from $10^3$ to $10^6$. For 32 serums from other sources, 96 per cent neutralized less than 100 embryo $l.d._{50}$ doses of NDV.

## SUMMARY

Determination of the lethal $dose_{50}$ neutralization indexes ($l.d._{50}$ NI) of 175 serums obtained from normal chickens without known history of contact with Newcastle disease virus resulted in zero values for 75.42 per cent, 1.32 to 5.89 values for approximately 20 per cent, and 6.76 to 14.79 for approximately 5 per cent of the serums. The $l.d._{50}$ NI was 10 or less for 174 (99.43%) of the serums.

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# Effects of Nasal Instillation of Virus Strains of Newcastle Disease Virus into the Cave Bat (Myotis Lucifugus)

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THE EGG-ADAPTED Newcastle disease virus (NDV) strains: California 11914; Colorado 1946, BAI 1; Delaware 1946, BAI 1; Kentucky 1946, BAI 1; Minnesota 1946, BAI 1; and Connecticut 1946, BAI 1, used for these studies were obtained, in 1946, from the Bureau of Animal Industry, Department of Agriculture, Washington, D. C. These strains were isolated by Bureau workers from infected chicken tissue from the corresponding states before the introduction of commercial live virus vaccines for Newcastle disease (ND).

The cultures had been carried in 10-day embryonating chicken eggs through six serial passages. In our laboratory, they were carried through six additional passages in 10-day embryonating White Leghorn eggs. Embryo passage of the California strain was continued through the twenty-fourth passage. Allantoamniotic fluid from several eggs dead of each strain was harvested and pooled separately for each strain. All strains of the virus were confirmed to be NDV by neutralization tests conducted in 10-day embryonating chicken eggs. The tests were performed according to the procedure recommended by the Bureau of Animal Industry.[1] Newcastle disease-immune chicken serum neutralized the virus in all cases while normal chicken serum had no effect. These six strains had been successfully transmitted to the cave bat, *Myotis lucifugus*, by intracerebral inoculation,[2] and the California strain had also been transmitted to the cave bat by intranasal instillation, by intraperitoneal inoculation, and by subcutaneous inoculation.[3]

One-hundredth cubic centimeter of pooled, infected allantoamniotic fluid from embryonating egg passages of all strains was instilled nasally into each of 6 cave bats. The bats were anesthetized with ether prior to the instillation. Within two to four days, bats injected with the California strain 11914 showed typical NDV symptoms: irritability, malaise, rhythmical contraction of muscles starting in the limbs and spreading over the entire body, paralysis of the hind legs, prostration, and death. There was no evidence of excessive salivation from pharyngeal paralysis, as seen in some other animals infected with the hamster-adapted NDV California strain 11914.[4]

TABLE 1—Bat Passage by Intranasal Instillation of California Strain 11914 of Egg-Adapted Newcastle Disease Virus

| Passage (No.) | No. bats inoculated | No. bats paralyzed, moribund, or dead | Min. to max. incubation period (days) |
|---|---|---|---|
| 1 | 8 | 7 | 2-4 |
| 2 | 8 | 6 | 2-4 |
| 3 | 8 | 6 | 2-4 |
| 4 | 8 | 6 | 2-4 |
| 5 | 8 | 5 | 2-4 |
| 6 | 8 | 8 | 2-4 |

Bats injected with the other five strains showed no typical ND symptoms after three attempts at exposure by this method. A total of 24 bats was used for each strain. From two to six days following inoculation, several bats were destroyed from each strain, the brains removed aseptically, ground to a 10 per cent suspension with physiological saline, and injected into the allantoic chamber of 10-day embryonating eggs. The eggs appeared normal and were discarded after seven days' incubation. This indicates that virus was present only in the brains of those bats receiving the California strain 11914.

TABLE 2—Titration of Newcastle Disease Virus, California Strain 11914, from Brains of Bats of the Sixth Intranasal Passage, Using Bats as the Test Host

| Virus dilutions | | | | Lethal doses |
|---|---|---|---|---|
| $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | |
| 3/5* | 3/5 | 4/5 | 1/5 | $10^{-4.5}$ |

*Numerator of fraction denotes the number of deaths; denominator denotes the number of bats injected.

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When nervous symptoms developed in the bats infected with the California strain, the brains were removed aseptically, ground with alundum, and diluted to a 10 per cent suspension with physiological saline. The bat brain suspension was inoculated into the allantoic chamber of 10-day embryonating eggs. All embryos injected with brains from the initial passage died within five days, whereas embryos, then on the second and third egg passages, died within forty-eight hours. Pooled allantoamniotic fluid from the dead eggs of this third passage was prepared. A neutralization test was done on the infected pool against NDV antiserum and normal chicken serum, using embryonating chicken eggs as the test host. The result of this test proved the virus to be that of ND.

The California strain 11914 of egg-adapted NDV was carried through six passages in the cave bat, as shown in table 1. Brain material for passage was chosen from bats destroyed when nervous symptoms developed. Quantities of 0.01 cc. of 10 per cent virus-bearing brain suspension from the preceding passage was instilled nasally throughout the six passages. These passages are being continued at the present time. A virus-neutralization test was conducted using brain suspension of the sixth intranasal bat passage with specific ND-immune and normal chicken serum, and with bats as the test host using the intranasal method of infecting the bats. Specific ND-immune serum completely neutralized the virus while normal chicken serum had no effect. The virus titrated $10^{-3.0}$ (table 2), according to the Reed-Muench method of calculation.[5]

## SUMMARY

The cave bat, *Myotis lucifugus*, is susceptible to the California strain 11914 of Newcastle disease virus by intranasal instillation. The California strain was carried six passages in the bat by intranasal instillation of infected bat brain suspensions. Newcastle disease virus infection could not be produced in bats by intranasal instillation of the Minnesota 1946, BAI 1; Colorado 1946, BAI 1; Kentucky 1946, BAI 1; Connecticut 1946, BAI 1; and Delaware 1946, BAI 1 strains of the virus. Three trials using intranasal instillation were made for these five strains.

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# Use of Sulfaquinoxaline as a Preventative Against Mild Outbreaks of Coccidiosis of Chickens Under Field Conditions

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EXTENSIVE LABORATORY and field experiments with various sulfonamides for the control of coccidiosis in chickens have been conducted by numerous investigators during the past ten years. In nearly all of this work, the effectiveness of the drug was tested either against artificial infection with massive doses of sporulated oöcysts or by administering the sulfonamide mixed with the mash to flocks exposed to natural infection in a heavily contaminated environment. Delaplane, Batchelder, and Higgins,[1] and later Grumbles, Delaplane, and Higgins,[2] reported that sulfaquinoxaline was effective as a prophylactic agent for coccidiosis when medicated mash was started before infection and was fed continuously or at intervals until the disease had run its course in the affected flocks. Again, the flocks used were exposed to heavy infection.

The essential difference between the experiments of Delaplane *et al.*[1] and others and those reported herein is that the flocks given sulfaquinoxaline as a prophylactic in these experiments had such a low degree of exposure to coccidiosis that the number of deaths in both treated and nontreated groups was so slight that mortality could not be used as the major criterion for evaluating the efficacy of the drug.

## MATERIALS AND METHODS

The trials were conducted on a farm where both cecal and intestinal coccidiosis had appeared annually for the past fifteen years. All of the chickens were White Leghorn females which were obtained as day-old chicks in three lots at four-week intervals. The chickens were brooded and reared on the floor, using wood shavings as litter, which was not stirred or changed during the experiment. When the chicks in each lot reached the age of 4 or 5 weeks, five equal groups were established. These groups were housed in pens under one roof but they were separated by wire partitions. There was no evidence of coccidiosis in any of the groups prior to the time the drug was administered.

The drug was mixed in the mash at the feed mill to insure an even distribution. The method of feeding consisted of providing both mash and grain. A daily record of the feed consumption was carefully maintained, from which the actual amount of drug in the total ration was calculated. To conserve space, a daily record of feed and drug consumed by each group of chickens is not included in this report, but the percentage amounts of sulfaquinoxaline in the mash and in the total ration during the course of the disease are summarized in tables 1 and 2.

Oöcyst counts were made, using the method previously described by Beach.[3] The feces were collected on sheets of heavy wrapping paper placed under each roost. The droppings were then well mixed and a representative sample taken for counting of oöcysts.

Sulfaquinoxaline determinations were made upon 1-ml. samples of whole blood diluted 1 : 20 with distilled water. A Cenco-Sheard-Sanford photelometer was employed for measuring the color reactions.

*First Trial.*—Beginning April 1, 1950, when the first lot of chicks reached an age of 5 weeks, one group was held as controls and four groups were continuously fed medicated mash containing 0.0166, 0.025, 0.05, and 0.1 per cent sulfaquinoxaline, respectively, as indicated in table 1. The treatment was continued until coccidiosis appeared and the results of the treatment were obtained.

The first evidence of coccidiosis appeared on April 17, 1947, when the appetite of the birds was observed to have decreased and a few bloody droppings were seen in pens 1 and 2. On April 19, 3 chickens were selected at random from the control group for autopsy. One chicken showed a slight amount of blood in the cecums, and many oöcysts and immature forms were found on microscopic examination of cecal scrapings. There was no evidence of intestinal coccidiosis. Lesions were not found in the other 2 birds. During the next four days (April 19-23), the disease became progressively worse in all of the pens but the infection was of a mild nature. The mortality resulting from the infection was too low to be of value in determining the efficacy of the various amounts of drug employed but, in spite of the mild nature of the disease, a difference in severity of the disease was noted by the amount of blood in the droppings, oöcyst counts, and general appearance (table

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The sulfaquinoxaline was supplied by Merck & Co., Rahway, N. J.

1). The chickens in the control pen (No. 1) were slightly depressed, and the droppings contained a moderate amount of blood. The most severely affected chickens were those in pens 2 and 3. These showed a marked depression, a drop in feed consumption, and considerable blood in the droppings. Chickens in pens 4 and 5 were less severely affected; approximately, 8 per cent of the birds in these respective pens showed dullness, inappetence, and a tendency to huddle. The greatest quantity of blood in the feces was seen in all pens on the sixth day of the disease, and after that the occurrence of blood rapidly diminished. By the tenth day, the droppings were free of blood.

Oöcyst counts made of the droppings collected over a period of seventeen hours from the evening of April 22 to the morning of April 23 showed that the greatest number of oöcysts was liberated by the chickens of pen 3, which received the least amount of the drug, while the birds of pen 4 shed the smallest number of oöcysts. The number of oöcysts found in the feces did not conform to the clinical observations in all instances, but the discrepancy may be due to the variations in the stages of the disease process among the groups at the time the samples were taken.

All surviving birds were examined two weeks after the last day that blood had appeared in the droppings. Differences in the appearance among the five groups of chickens were not observed. The medicated mash was, therefore, discontinued, and 20 birds from each pen were challenged with 100,000 sporulated oöcysts of *Eimeria tenella*. No evidence of coccidiosis appeared in the challenged chickens or in their pen mates during the succeeding two weeks.

*Second Trial.*—The procedure of the second trial was the same as in the first trial, except that the concentrations of sulfaquinoxaline in the mash of the four treated groups were changed to 0.1, 0.066, 0.05, and 0.033 per cent, respectively, and the medicated mashes were given for seven-day periods, with intervening seven-day periods during which regular mash was fed (table 2).

Both cecal and intestinal coccidiosis occurred in the flock, but the two types of infections differed in the time of their appearance. Cecal coccidiosis occurred four weeks from the time the experiment was begun and on the fourth day of a week during which regular mash was being fed. The results are summarized in table 2.

The first sign of cecal coccidiosis, which consisted of the appearance of blood in the droppings of the chickens of groups 1, 2, and 3, was noted on the morning of May 8, 1947. Judging from the amount of blood and the appearance of the birds, the controls (group 1) appeared least affected, whereas the birds of group 3 were most severely affected. Three days later, a few bloody droppings appeared in pens 4 and 5 but, forty-eight hours later, the feces in these pens as well as in pens 1, 2, and 3 were free of blood again.

There was no significant difference in the mortality during the first week (May 6-12) of the appearance of coccidiosis when all groups were receiving the regular mash; however, in the following week (May 13-19), when the treated groups were again placed on medicated mashes, the mortality increased in all pens (table 3). Autopsy of some of the birds of each group that died during these periods showed lesions of cecal coccidiosis in the treated groups and both cecal and intestinal forms of the disease in the nontreated control group. The results of the examination of feces passed on the evening of May 18 and the morning of May 19 indicated that the controls were still shedding a few oöcysts, while the treated chickens were not (table 2).

Although no further clinical cecal and intestinal coccidiosis was observed in the control group after the period May 13-19, symptoms of the disease reappeared in the treated groups on May 24, five days after the birds

**TABLE I—Severity of Infection and Sulfaquinoxaline (SQ) Blood Levels of Five Groups of Chickens Fed a Grain-Mash Ration Containing 0.0166, 0.025, 0.05, and 0.10 Per Cent SQ in the Mash Continuously for Forty-Two Days**

| Group (No.) | No. birds | SQ in mash (%) | SQ in total ration (Ave. %)* | Severity of infection** | No. dead April 17-26 | Oöcyst per Gm. feces April 22-23 | Immunity† |
|---|---|---|---|---|---|---|---|
| 1 | 578 | 0.000 | .000 | ++ | 3 | 17,400 | Immune to *Eimeria tenella* |
| 2 | 622 | 0.025 | .013 | +++ | 4 | 21,600 | Immune to *E. tenella* |
| 3 | 633 | 0.0166 | .008 | +++ | 6 | 53,800 | Immune to *E. tenella* |
| 4 | 635 | 0.050 | .025 | + | 3 | 10,300 | Immune to *E. tenella* |
| 5 | 633 | 0.100 | .049 | + | 2 | 18,000 | Immune to *E. tenella* |

*Calculated from daily records of mash and grain fed to the chickens during the course of the disease April 17 to 24. **Severity of infection of cecal coccidiosis was based on appetite, general appearance, and amount of blood in droppings during course of the disease, as determined by inspection. †Twenty birds in each pen challenged with 100,000 sporulated oöcysts two weeks after the last day blood was seen in droppings.

had been taken off their respective mashes. Anorexia was a prominent symptom which became progressively worse during the next two days. The birds in groups 2, 3, and 4 were cecal material, and, in almost all cases, the walls of the small intestine were markedly thickened and petechiated. No single species of coccidia could be incriminated in the mixed

**TABLE 2—Effect of Sulfaquinoxaline (SQ), Given Continuously in the Ration for Seven Days Followed by an Intervening Period of Seven Days when Regular Mash Was Given, upon Cecal and Intestinal Coccidiosis**

| Group (No.) | No. birds | SQ in Mash (%) | SQ in Total ration** (%) | Severity of disease* 1st outbreak | Severity of disease* 2nd outbreak | Oöcysts per Gm. feces May 18-19 | Oöcysts per Gm. feces May 30-31 | Immunity† |
|---|---|---|---|---|---|---|---|---|
| 1 | 625 | 0.00 | 0.000 | C I ++ | nil | 230 | 0 | Immune to *Eimeria tenella* |
| 2 | 622 | 0.100 | 0.047 | C ++ | C I ++ | 0 | 1,635 | Immune to *E. tenella* |
| 3 | 622 | 0.066 | .030 | C +++ | C I ++ | 0 | 2,095 | Immune to *E. tenella* |
| 4 | 626 | 0.050 | .024 | C + | C I +++ | 0 | 4,735 | Immune to *E. tenella* |
| 5 | 621 | 0.033 | .016 | C + | I + | 0 | 0 | Immune to *E. tenella* |

*Type and severity of infection based upon autopsy, feed consumption, bloody droppings, and condition of the groups on inspection. C = cecal coccidiosis, I = intestinal coccidiosis. First outbreak May 8 to 19. Second outbreak May 24 to 30. **Average sulfaquinoxaline in per cent, based upon calculation of mash and grain consumed during course of first and second outbreaks. †Fifteen chickens in each pen inoculated with 100,000 sporulated oöcysts of *Eimeria tenella* two weeks from the first day that blood was seen in droppings of second outbreak. Not tested for immunity to intestinal species.

markedly depressed, and the droppings were watery and blood flecked. These symptoms were most prominent in group 4. No blood was found in the droppings of group 5 during this period, and the only symptom observed was a slight depression which was evident on May 25 and 26. In the meantime, the control chickens remained active and alert. The disease continued until May 30, four days after resumption of the medicated mash. Thereafter, the birds improved rapidly and, by June 1, they appeared normal with respect to both appetite and activity.

Postmortem examination of the birds that died during the period of the relapse showed evidence of both intestinal and cecal coccidiosis. The cecums contained caseous cores or small amounts of blood admixed with the infection. Microscopic examination of the intestinal contents revealed a large number of developing coccidial forms.

On June 8, approximately two weeks from the first day that the blood was seen in the droppings in the second outbreak, 15 birds of each group were inoculated orally with 100,000 sporulated oöcysts of *E. tenella*. No evidence of cecal coccidiosis was seen in the inoculated chickens or in their pen mates during the two-week observation period. The inoculation of 4 susceptible controls with the same material caused an acute form of disease in all of the birds by the fourth day, followed by death of 2 birds on the fifth day.

*Third Trial.*—When the third lot of chicks was 4 weeks old (on May 10), it was divided into five groups, each containing approximately

**TABLE 3—Mortality of One Nontreated Group and Four Groups of Chickens Treated with 0.1, 0.066, 0.05, or 0.033 Per Cent Sulfaquinoxaline (SQ) in the Mash for Seven Days at Weekly Intervals**

| Group (No.) | Period* | | | | | | | | | | Total mortality |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | May 6-12** | | May 13-19 | | May 20-26† | | May 26-June 2 | | June 3-9 | | |
| | Treat. | Mort. | Treat.‡ | Mort. | Treat. | Mort. | Treat. | Mort. | Treat. | Mort. | |
| 1 | None | 3 | None | 12 | None | 1 | None | 1 | None | 0 | 17 |
| 2 | None | 2 | 0.1% SQ | 10 | None | 5 | 0.1% SQ | 11 | None | 0 | 28 |
| 3 | None | 2 | 0.066% SQ | 3 | None | 7 | 0.066% SQ | 13 | None | 0 | 25 |
| 4 | None | 0 | 0.05% SQ | 8 | None | 11 | 0.05% SQ | 29 | None | 0 | 48 |
| 5 | None | 3 | 0.033% SQ | 4 | None | 3 | 0.033% SQ | 3 | None | 0 | 13 |

*Treatment and mortality in the five groups of chickens during the seven-day periods shown. **First outbreak, May 8 to 19. †Second outbreak, May 24 to 30. ‡Per cent sulfaquinoxaline in the mash of the mash-grain ration.

630 chicks. The treatment consisted of either 0.1 per cent or 0.2 per cent of sulfaquinoxaline in the mash, which was given for two or three days of the week followed by a five- or four-day period on a regular mash, as shown in table 4.

The intermittent feeding of medicated mash was begun on May 10 and continued until June 22, an elapsed period of forty-four days. The attendant noted that the chickens in groups 2 and 3, which received the 0.2 per cent sulfaquinoxaline mash, showed a marked distaste for the medicated feed when it was set before them, but the 0.1 per cent sulfaquinoxaline mash was consumed readily. Determinations of sulfaquinoxaline blood concentrations following forty-eight hours on the respective medicated mashes showed that higher sulfaquinoxaline blood levels were obtained with the 0.1 per cent medicated mash than with the mash containing 0.2 per cent of the drug (table 4). It was apparent, therefore, that mashes containing 0.2 per cent of sulfaquinoxaline were not consumed in normal quantities by the chickens.

A mild form of intestinal coccidiosis due to *Eimeria maxima* was detected in the flock on June 8, during the fifth week of the experiment. The results, as summarized in table 4, show that both the 0.2 and 0.1 per cent sulfaquinoxaline mashes appeared to exert a marked coccidiostatic effect; however, the latter concentration proved to be more effective than the former. The disease was still evident in the control pen after June 15. In this pen, 25 per cent of the birds continued to show listlessness and inappetence, but the four groups of chickens which were on the medicated mash appeared normal. At no time was blood found in the feces of any group.

On June 22, all of the birds appeared bright, alert, and active. Inasmuch as oöcysts of *E. maxima* were not available to determine the degree of immunity in the flock to this species, it was decided to determine whether enough undetected coccidiosis caused by *E. tenella* may have been present in the chickens to immunize against this species. For this purpose, 3 birds, picked at random from each of the five pens, were challenged orally with 130,000 oöcysts of *E. tenella*. No symptoms were observed and none of the chickens died of *E. tenella* infection, but they were shedding oöcysts, and 5 chickens (3 from pen 3 and 1 bird from each of pens 1 and 2, respectively) showed lesions of the disease upon autopsy. It is apparent that the birds in all five groups had acquired enough *E. tenella* infestation at some time during their lives on the ranch to make them wholly or partially immune to this protozoon.

## DISCUSSION

Reported herein are the results of a field trial with sulfaquinoxaline given in various concentrations continuously and at intervals to three flocks, comprising 9,380 chickens on the same poultry farm. The drug was given in the mash, but the flocks were fed a mash and grain ration, and the percentage of sulfaquinoxaline in the total ration was computed from the daily records of the

TABLE 4—Severity of Infection, Mortality, Oöcyst Output, and Sulfaquinoxaline (SQ) Blood Concentrations in Five Groups of Chickens of Trial 3 Which Were Fed a Mash-Grain Ration Containing 0.1 or 0.2 Per Cent SQ in the Mash for Two or Three Days Followed by the Regular Mash for the Following Five or Four Days, Respectively

| Pen (No.) | Treatment | Severity of infection* | Mortality | | Oöcysts per Gm. feces | Mg. SQ** 100 cc. blood | |
|---|---|---|---|---|---|---|---|
| | | | June 8-15 | June 16-23 | June 9-10 | Mean | Range |
| 1 | None (controls) | +++ | 15 | 6 | 35,414 | ... | ... |
| 2 | 0.2% on 2-5 | + | 4 | 2 | 5,384 | ... | 0 to 1.02 |
| 3 | 0.2% on 3-4 schedule | + | 1 | 1 | 770 | ... | 2.09 |
| 4 | 0.1% on 2-5 schedule | —<br>+ | 2 | 1 | 0 | ... | 0 to 1.40 |
| 5 | 0.1% on 3-4 schedule | —<br>+ | 0 | 1 | 0 | ... | 2.22 |

*Severity of infection based upon symptomatology on inspection. **Sulfaquinoxaline blood concentrations of 20 chickens following forty-eight hours on the respective medicated mashes. Determinations made prior to infection of flock.

relative amounts of mash and grain consumed. Although coccidiosis had occurred annually and had been a serious problem on this farm for the past fifteen years, the severity of the infection during this trial was sufficiently mild so that treatment could not be evaluated upon the mortality alone.

In a mild outbreak of cecal coccidiosis, the continuous feeding of a medicated mash in a ration which furnished 0.025 per cent sulfaquinoxaline during the course of the disease was found to be beneficial. The clinical symptoms of depression, bloody droppings, and oöcyst production in the group receiving this level were noticeably minimized. A ration which contained 0.049 per cent sulfaquinoxaline of the total ration was found to be slightly more effective. Grumbles, Delaplane, and Higgins[2] reported that a sulfaquinoxaline mash containing 0.0125 per cent of the drug given continuously was effective in decreasing the mortality in *severe* outbreaks of *E. tenella* infections. The results of this field trial showed that rations which supplied an average of 0.008 or 0.013 per cent sulfaquinoxaline in the total ration were of little or no value in reducing the clinical symptoms in a *mild* outbreak of coccidiosis caused by *E. tenella*. It is evident therefore that, because of the mild nature of the disease, treatment by the continuous method as a preventative measure against this infection was unnecessary and consequently uneconomical.

In another trial, administration of sulfaquinoxaline in the mash to chickens for periods of seven days, with intervening periods of seven days when regular mash was fed, was found to be of some value in cecal and intestinal coccidiosis when given in the mash at a level which supplied from 0.015 to 0.018 per cent of the drug in the total ration. The maintenance of such concentrations of the drug in the ration was found to reduce the clinical symptoms, reduce the mortality from both cecal and intestinal coccidiosis, and enable the chickens to become immunized against infections to which they were exposed after withdrawal of the sulfaquinoxaline mash. Higher concentrations of the drug in the mash, which supplied 0.047, 0.030, and 0.024 per cent of the drug in the total ration, respectively, appeared to be too highly coccidiostatic. This apparently precluded immunity production, as shown by a relapse of the infection which occurred three to five days after the medicated mash was withdrawn. A study of the mortality also suggests that the interval of seven days between treatments was too long, because the highest death rates were found during the period the birds were treated. The drug apparently protected the birds completely while treated and soon thereafter, because, when the medicated mash was withdrawn, the disease reappeared and was too well advanced to be affected by the drug when treatment was resumed again. There was no evidence to indicate that intestinal coccidiosis was present in the treated groups at the time of the first outbreak; however, it is impossible to ascertain whether the drug prevented the development of the parasite during the period of treatment or whether the spread of intestinal coccidiosis from the control group to the other pens was slower than the cecal form of the disease.

It is of interest to compare the results of trials 1 and 2, which involved treating a mild form of coccidiosis caused by *E. tenella* under field conditions. Sulfaquinoxaline concentrations in the total ration of 0.013 per cent were not effective in controlling the clinical symptoms associated with the disease in the first trial, but a slightly higher drug concentration of 0.016 per cent in trial 2 was not only effective in alleviating the symptoms but did not retard immunity production. Higher concentrations of the drug in the total rations of 0.025 and 0.024 per cent in these trials, respectively, were very effective against clinical manifestations of the infection, however the latter concentrations interfered with production of an immunity. It is apparent that the concentration of drug employed is bound within narrow limits for its maximum effectiveness against the clinical manifestations and production of an immunity toward this protozoon.

Sulfaquinoxaline, when given in the mash at the rate of 0.1 per cent or 0.2 per cent in a mash-grain diet for two or three days of the week, was found to be coccidiostatic against a mild infection of coccidiosis caused by *E. maxima*. The 0.1 per cent sulfaquinoxaline which furnished 0.035 to 0.05 per cent of the drug in the total ration appeared to be more effective than the mash containing a higher concentration, and, since the disease was of a mild nature, the relative efficiency of medication for two or three days of the week could not be ascer-

tained. The medicated mash on these schedules markedly reduced the clinical symptoms, reduced mortality, and shortened the duration of the disease. When the sulfaquinoxaline concentration was increased to 0.2 per cent in the mash, the treatment was less effective, because the medicated feed was sufficiently distasteful to the birds to defeat, partially, its therapeutic purpose. The average concentration of sulfaquinoxaline in the blood of 20 chickens, picked at random following forty-eight hours of medication, was less than in chickens which received the 0.1 per cent sulfaquinoxaline mash.

## SUMMARY

The results of three trials, with sulfaquinoxaline administered to chickens in a mash as a prophylactic agent against an impending infection with coccidiosis, indicated that the drug was effective in alleviating clinical manifestations in chickens infected with mild cecal and some forms of intestinal coccidiosis. The effective dosage for alleviating symptoms of cecal coccidiosis by the continuous method of treatment, however, appeared to be greater than the amount which has been reported as effective in decreasing the mortality from more severe forms of the disease.

The disease in the trials as reported herein was not sufficiently serious to warrant treatment by preventive methods which are widely recommended at the present time.

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# Botulism in the Mouse, Mink, and Ferret with Special Reference to Susceptibility and Pathological Alterations

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BOTULISM HAS continued to cause heavy losses on fur farms in recent years. In the most of these cases, however, the diagnosis has been based almost solely upon the symptoms in conjunction with the circumstances under which the disease has occurred.[1, 3, 5, 15, 18, 19, 21, 25] Quortrup and Holt[17] reported three outbreaks of botulism in mink with a mortality of 90 per cent. Typing with specific antiserums showed that these cases were due to type C toxin. Hall and Stiles[4] reported an outbreak in mink with a 96 per cent fatality rate due to *Clostridium botulinum*, type A. Pyle and Brown[16] reported botulism in Silver Foxes and, on typing of filtrate from the suspected food boxes against specific antiserums, it was concluded that the outbreaks were caused by type A toxin.

Shoop,[23, 24] using a type A culture, could cause death in Silver Foxes by injecting 50,000 to 100,000 mouse minimum lethal doses (m.l.d.) of filtered culture subcutaneously. The filtrate contained $10^4$ to $10^7$ mouse m.l.d. per cubic centimeter, and ten times this quantity given *per os* did not produce any apparent injury. However, 2.5 x $10^6$ mouse m.l.d. of unfiltered culture *per os* was held responsible for the death of 1 of the foxes after five days. Shoop,[22] using type A culture, found that 2 x $10^5$ mouse m.l.d. *per os* did not cause symptoms in mink, while $10^4$ m.l.d., subcutaneously, killed this species in twenty-four hours. The mouse m.l.d. of this culture represented 0.0001 cc. This author also reported that, after receiving *botulinum* toxin *per os*, a slight degree of immunity occurred in the mink but not enough to produce a refractivity to subcutaneous injection. Quortrup and Gorham[20] reported an investigation which showed that mink and ferrets were moderately susceptible to the toxins of *Clostridium parabotulinum*, types A and B, and highly susceptible to the toxin of *Cl. botulinum*, type C, whereas they appeared to be immune to type E.

Various authors claim botulism to be of little or no importance in furbearing animals.[21] Many reports of heavy losses in these animals are, however, presumed to be due to botulism. The way the food is sometimes handled might certainly provide excellent conditions for toxin production by *Cl. botulinum* and *Cl. parabotulinum*.

In consideration of the facts and data cited and in view of the availability of suitable animals and toxins, a study was undertaken to determine the susceptibility of mink and ferrets to the purified toxin of the types A and B of *Cl. parabotulinum*. Critical observations were also possible on the acute phase and postmortem changes in the poisoned animals.

## EXPERIMENTAL MATERIALS AND METHODS

Quantities of purified toxins, types A and B, were received in April, 1948, through the courtesy of Dr. Carl Lamanna of Camp Detrick, Md.[7, 8, 9] The toxins were subsequently stored in rubber-stoppered glass bottles in the refrigerator at 4 C. for five months before the experiment was undertaken. Immediately before use, the necessary amount of toxin was weighed out and suspended by triturating with saline (pH about 4.2) in sterile mortars. Saline was used also for making dilutions. Because of a slight tendency toward settling, the suspensions were shaken well immediately before animal introduction. The mice used for the titration of toxin were from two different strains of the same stock. The mink and ferrets used in the experiments were all raised at the same fur ranch and were approximately six months old when the study was initiated.

*Determination of the Minimum Lethal Dose for the Mouse.*—Since the work covered a considerable period of time, the potency of the

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toxins was tested repeatedly at certain intervals, both by the peritoneal and oral routes, on 20-Gm. white mice. A group of 6 mice was used for each quantity of toxin and one group, serving as control, was given an equal amount of saline by each route. Various dilutions of toxin were given to each mouse in 0.1-cc. quantities, intraperitoneally; 1.0 cc. by a stomach tube.

The m.l.d. was established as the smallest quantity of toxin which would kill all the mice within the group in seventy-two hours (table 1). The spread of the m.l.d. range in the mouse is not unusual, in view of our preliminary results as well as those of Shoop.[21, 22, 23, 24]

Following administration of large doses (1,000 m.l.d. or more) of toxin to mice, symptoms developed rapidly followed shortly by death. Following administration of smaller doses (100 m.l.d. or less), the development of symptoms was delayed and the intoxication progressed more slowly. Labored abdominal breathing and convulsions preceding death were the classical expressions of the poisoning.

*Susceptibility of Ferrets to Purified Parabotulinum Toxin, Types A and B, Intraperitoneally and Per Os.*—Fourteen groups of ferrets, consisting of 3 males and 3 females in each group, were given purified *botulinum* toxin in saline, intraperitoneally. Four groups received the type A toxin and ten groups received the B toxin. Three additional groups serving as controls were given saline by the same route (table 2).

Sixteen similar groups of ferrets were given purified *botulinum* toxin in saline *per os*. The toxin was administered under light ether anesthesia by means of a 1-cc. glass pipette as a stomach tube attached to a syringe. Ten of these groups received the type A toxin and seven groups received the type B toxin. Five additional groups serving as controls were given saline only (table 2).

The animals were not fasted previous to treatment, and food was accessible to them immediately after treatment.

Symptoms and Issue in Ferrets.—All animals receiving 0.01 mg. or more of A toxin or 2 mg. or more of B toxin, intraperitoneally, developed symptoms and all died. The symptoms started twelve to forty-eight hours after inoculation and consisted of squinting of the eyelids, dullness, urine soiling of the fur of the belly and rear extremities, ataxia, paralysis, and prostration. The animals lost weight very rapidly from the time symptoms appeared until fatal issue. Death ensued from one to three days after inoculation, and the course of the intoxication was, to a considerable extent, independent of the dosage of toxin given.

Ferrets given toxin *per os* showed great variability in response. As little as 10 mg. of A toxin and 5 mg. of B toxin sometimes caused symptoms and death, while dosages as large as 60 mg. of A toxin or 40 mg. of B toxin did not induce marked symptoms of poisoning in other individuals. As after parenteral injection, symptoms appeared in twelve to seventy-two hours and death ensued in from to one to seven days. In all these experiments, the symptoms appeared sooner after administration of A than B toxin, and the course of the intoxication was slightly shorter for A than for B toxin. The symptoms were identical with A and B toxins by both routes of administration.

Pathological Changes.—Necropsy of dead animals was performed immediately after death. On gross examination, all revealed congestion of the liver, spleen, and kidney; subcapsular hemorrhage of the spleen; collapse of the lungs; distension of the bladder, gall bladder, and lower part of intestine; and, in some instances, petechial hemorrhage of the thymus.

Tissues for microscopic study were fixed in neutralized 10 per cent formalin and stained with hematoxylin-eosin.

The same changes were observed in all dead animals, although those surviving the longest times showed more marked involvement.

The liver sections revealed parenchymal congestion and hyperemia with distension of the capillaries and veins. Some hemorrhages were also seen. The lungs showed alveolar collapse and extensive hyperemia with some hemorrhage. The kidneys manifested hyperemia with hemorrhages and degeneration of the tubular epithelium. A few hemorrhages were seen in some sections of the cerebellum. The spleens revealed a striking picture, most marked in those animals from group 2 which had survived four to seven days before succumbing. This organ was hyperemic with numerous subcapsular and follicular hemorrhages. The spleens of the animals which died twenty-four hours after inoculation were congested with well-defined necrosis of the centers of the lymphoid follicles. There were signs of clumping of the reticulum cells into islands, and some giant cells.

The spleens of animals which died seventy-two or more hours after inoculation exhibited hyperplasia of the mononuclear cells in the centers of the follicles together with a thinning out of the lymphocytes, as well as a definite clumping of reticulum cells and the appearance of relatively large numbers of giant cells. An inflammatory reaction could not be detected in any of the other tissues examined.

*Supplementary Experiment 1.*—To exclude the possibility that failure to develop intoxication was the result of vomition soon after administration of the toxin, one group of ferrets, 3 males (weight 1,520 to 1,580 Gm.) and 3 females (weight 650 to 790 Gm.), were each given 40 mg. of type A toxin *per os* and placed in a wire-bottomed cage over white paper. The toxins were diluted in 4 cc. saline to which carmine was added. One additional group serv-

ing as controls was given saline mixed with carmine only. The animals were watched closely for forty-eight hours and evidence of emesis was not seen in any instance. The control animals did not show any symptoms, while 2 females in the group which received toxin showed symptoms forty-eight hours after treatment. Both of the latter died within seventy-two hours after treatment, with typical symptoms of urine soiling, ataxia, and paralysis. One of the males showed symptoms of eye squinting and of dullness six days after treatment but recovered.

*Supplementary Experiment 2.*—To determine the effect of the acidity of the stomach upon the course of the poisoning, 2 males (weight 1,880 and 2,020 Gm.) and 2 females (weight 700 and 730 Gm.) were each given 40 mg. of type A toxin *per os* diluted in a mixture of one part saline and one part 50 per cent $Al_2O_3$. The 2 females showed characteristic symptoms of *botulinum* poisoning three days after administration and died in prostrate position during the fourth day following receipt of the toxin. The 2 males remained apparently healthy during the observational period of one week.

*Supplementary Experiment 3.*—To ascertain whether the environmental temperature might have a marked influence on *botulinum* poisoning, two groups of ferrets, 1 male and 1 female in each group, were each given 40 mg. of type A toxin *per os*. This experiment was carried out during the period November 19, 1948 to November 24, 1948, at which time the atmospheric temperature ranged from 22 to 46 F. One group was kept outside while the other group was kept inside at a temperature of 80 to 85 F.

Two days after treatment, the females in both groups showed symptoms of dullness and squinted eyes. On the third day, the female kept outside was prostrate and it died on the fourth day, while the female kept inside recovered by the sixth day.

*Supplementary Experiment 4.*—Two groups of ferrets, consisting of 1 male and 2 females in each group, were given 40 mg. of type A toxin each by stomach tube. The males weighed 1,150 and 1,200 Gm. and the females 490 and 640 Gm. One group had been fasted for forty-eight hours, while the other group had not been fasted before treatment with toxin.

One female in each group died three days after introduction of the toxin. Prostration preceded death in each.

*Supplementary Experiment 5.*—To determine whether spoiled food had any influence on the pathology of the intestinal tract of poisoned animals, two groups of ferrets, 2 males (weight 1,130 and 1,200 Gm.) and 2 females (weight 600 and 670 Gm.), were given decomposed meat after being fasted for forty-eight hours. To the spoiled meat given one group, 40 mg. of type A toxin was added. None of these animals developed any symptoms.

*Susceptibility of Mink to Purified Parabotulinum Toxin Types A and B, Intraperitoneally and Per Os.*—Nine pairs of mink were injected intraperitoneally with purified *botulinum* toxin in saline. Three pairs received the type A toxin and six pairs the type B toxin. Two additional pairs serving as controls were given saline only by the same route (table 3). Twelve mink received purified A toxin suspended in saline and mixed with the food. Two additional mink serving as controls were given saline mixed with the food (table 3). Eleven mink received purified B toxin suspended in saline and mixed with the food (table 3). Each animal was kept in a separate pen.

TABLE I—Titration of the Minimum Lethal Dose of Types A and B Toxins in Mice

| Route | Date | Type of toxin | m.l.d./mouse |
|---|---|---|---|
| i.p | 9/20/48 | A | 0.000001 mg. |
| i.p. | 9/25/48 | A | 0.00001 mg. |
| i.p. | 12/10/48 | A | 0.0000001 mg. |
| i.p | 2/16/49 | A | 0.00001 mg. |
| i.p. | 7/16/49 | A | 0.000001 mg. |
| i.p. | 9/11/48 | B | 0.00001 mg. |
| i.p. | 12/ 6/48 | B | 0.0000001 mg. |
| i.p. | 2/16/49 | B | 0.000001 mg. |
| i.p. | 7/16/49 | B | 0.000001 mg. |
| *Per os* | 9/29/48 | A | 0.1 mg. |
| *Per os* | 10/23/48 | A | 0.01 mg. |
| *Per os* | 11/ 8/48 | A | 0.01 mg. |
| *Per os* | 9/16/49 | A | 0.01 mg. |
| *Per os* | 9/13/48 | B | 0.1 to 0.01 mg. |
| *Per os* | 10/23/48 | B | 0.01 mg. |
| *Per os* | 11/ 8/48 | B | 0.01 mg. |
| *Per os* | 7/16/49 | B | 0.01 mg. |

i.p. = intraperitoneal.

*Symptoms and Issue in Mink.*—None of the animals receiving 0.01-0.5 mg. of type A toxin, intraperitoneally, had developed any symptoms eight hours after injection, but all of them, except the controls, were found dead in prostrate position eighteen hours after injection.

All animals receiving 0.1 mg. or more of the type B toxin, intraperitoneally, manifested symptoms of dullness, ataxia, and finally paralysis during the day after inoculation. Each died in prostrate position from eighteen to seventy-two hours after inoculation.

All animals receiving 40 or 50 mg. of A toxin *per os* showed slight symptoms during the fourth day after administration of toxin but recovered. One animal which had received 60 mg. was found dead in prostrate position the morning after feeding the toxin. The other animal in this group developed symptoms of ataxia after two days but recovered. The animal which received 70 mg. of A toxin *per os* died after four days, without having shown any symptoms, and the 2 animals receiving 80

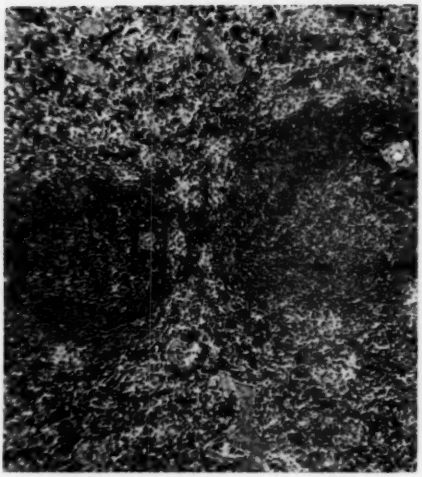

Fig. 1—Normal mink spleen. x 75. Large, well-defined malpighian bodies with lighter germination centers.

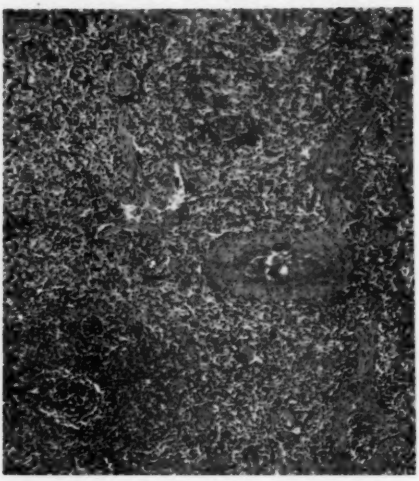

Fig. 2—Spleen of mink dead eight days after consumption of botulinum toxin type B. x 75. Ill-defined malpighian bodies.

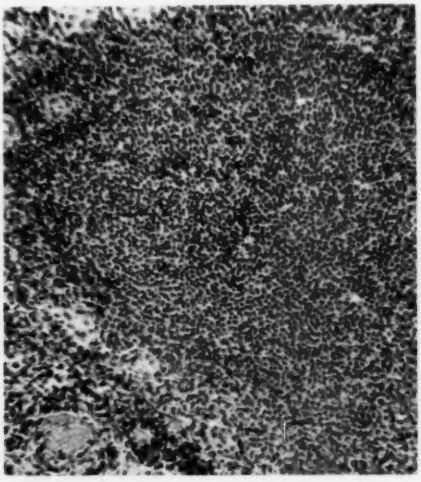

Fig. 3—Normal mink spleen. x 150. Large, well-defined malpighian body with relatively numerous lymphocytic elements.

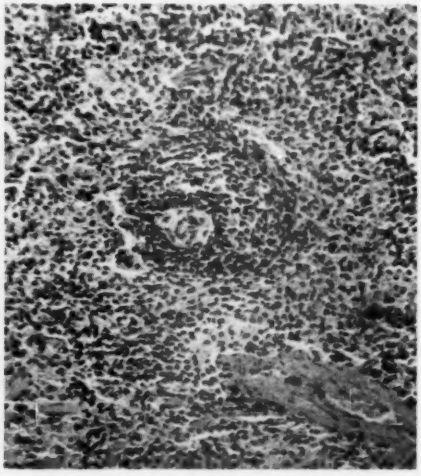

Fig. 4—Spleen of mink dead eight days after consumption of botulinum toxin type B. x 150. Remnant of a malpighian body and relatively few lymphocytic elements.

mg. were prostrate eight days after treatment, at which time they were killed.

All the animals receiving 20 mg. or more of B toxin *per os* developed symptoms three days after consumption of the toxin. The symptoms were dullness, squinting of the eyes, paralysis of the membrana nictitans, urine soiling, and ataxia. The animals which received 50 and 60 mg. of B toxin recovered, while all the others died from four to eight days after consuming the toxin.

*Pathological Changes.*—Necropsies were performed within one to six hours after death. The gross lesions were the same as described for the ferrets. The alterations in the mink, as in the ferrets, were more marked in animals which survived longest.

Organ tissues were prepared for microscopy in the same manner as the ferret tissues. The histological alterations were qualitatively the same in all animals. The livers showed hyperemia and hemorrhage with marked distension of the capillaries and veins and some degeneration of the parenchyma. The lungs exhibited collapse of the alveoli and diffuse, rather extensive hyperemia throughout. In the cerebellum of some of the animals, a few, relatively small hemorrhages were found.

The spleen of animals dead one to two days after administration of both toxins was filled with blood and extensive subcapsular hemorrhages were present. Some of the follicles seemed to be necrotic, others revealed a perifollicular hemorrhage.

The spleens of mink dead on the fourth day after poisoning exhibited prominent lesions. The follicles were indistinct and, as a result of mononuclear cell hyperplasia, very few lymphocytes remained. In some places, there was considerable destruction and only fragments of the follicles and reticulum persisted. Subcapsular hemorrhages as well as hemorrhages

**TABLE 2—Determination of Susceptibility of Ferrets to Botulinum Toxin Types A and B by the Intraperitoneal Route and Per Os**

| A Toxin | | | | | B Toxin | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group* | Mg. toxin | Vol. saline in inoc. | Route | Result | Group* | Mg. toxin | Vol. saline in inoc. | Route | Result |
| 1 | 0.001 | 1.0 cc. | i.p. | No symptoms | 1 | 0.001 | 1.0 cc. | i.p. | No symptoms |
| 2 | 0.010 | 1.0 cc. | i.p. | Dead 24-72 hr. after inoculation | 2 | 0.010 | 1.0 cc. | i.p. | No symptoms |
| 3 | 0.100 | 1.0 cc. | i.p. | Dead 24 hr. after inoculation | 3 and 4 | 0.100 | 1.0 cc. | i.p. | No symptoms |
| 4 | 1.000 | 1.0 cc. | i.p. | Dead 24 hr. after inoculation | 5 and 6 | 1.000 | 1.0 cc. | i.p. | No symptoms |
| Control | .... | 1.0 cc. | i.p. | No symptoms | 7 | 2.000 | 2.0 cc. | i.p | Dead in one to three days after inoculation |
| 1 | 0.100 | 1.0 cc. | *Per os* | No symptoms | 8 | 3.000 | 3.0 cc. | i.p. | Dead in one to three days after inoculation |
| 2 | 1.000 | 1.0 cc. | *Per os* | No symptoms | 9 | 4.000 | 4.0 cc. | i.p. | Dead in one to three days after inoculation |
| 3 | 5.000 | 1.0 cc. | *Per os* | No symptoms | 10 | 5.000 | 5.0 cc. | i.p. | Dead in one to three days after inoculation |
| 4 | 10.000 | 1.0 cc. | *Per os* | One died one day after toxin administration | Control | .... | 1.0 cc. | i.p. | No symptoms |
| 5 and 6 | 20.000 | 2.0 cc. | *Per os* | No symptoms | Control | | 5.0 cc. | i.p. | No symptoms |
| 7 and 8 | 30.000 | 3.0 cc. | *Per os* | Three females in group 7 died four days after, 1 female in group 8, one day after toxin administration | 1 | 1.000 | 1.0 cc. | *Per os* | No symptoms |
| 9 | 40.000 | 4.0 cc. | *Per os* | One female died one day after toxin administration | 2 and 3 | 5.000 | 1.0 cc. | *Per os* | Three males and 2 females dead three to seven days |
| 10 | 60.000 | 6.0 cc. | *Per os* | No symptoms | 4 and 5 | 10.000 | 2.0 cc. | *Per os* | Two females dead four to seven days |
| Control 1 | .... | 1.0 cc. | *Per os* | No symptoms | 6 | 20.000 | 4.0 cc. | *Per os* | One female dead one to two days |
| Control 2 | .... | 3.0 cc | .... | No symptoms | 7 | 40.000 | 4.0 cc. | *Per os* | Killed nine days |
| Control 3 | .... | 6.0 cc. | .... | No symptoms | Control 1 | .... | 1.0 cc. | *Per os* | No symptoms |
| | | | | | Control 2 | .... | 4.0 cc. | *Per os* | No symptoms |

*Each group consisted of 3 males and 3 females. Weight range in grams: males, 900 to 1400, females, 700 to 1000. i.p. = intraperitoneal.

in the pulp and follicles were seen. Quite a few multinuclear giant cells were present. In the spleen of the mink dead eight days after receiving the poison, only remnants of lymphocytes could be detected and very little of the normal lymphatic architecture seemed to remain (fig. 2, 4, 6). All follicles were indistinct, the lymphocytes having been replaced by mononuclear cells (fig. 6).

The same type of alteration as seen in the spleens was present to some extent in the lymph nodes (fig. 8). All lymph nodes were hyperemic, and the blood pigment was largely taken up by the reticulum cells of the lymph sinuses.

The lymph nodes of the animals dead at the fourth and fifth days after poisoning were characterized by almost complete disappearance of the lymphocytes, with a resultant very light cortex and clear centers in some cases.

Scrutiny of the adrenal glands did not reveal any marked alterations. Some signs of hemorrhage and swelling of the cells in the cortex seemed to be present, but conclusions could not be drawn as to the significance of these findings.

Of the animals which received 40 to 50 mg. of B toxin and which showed symptoms, 2 recovered. They were killed in apparently healthy condition on the tenth day. Neither gross nor microscopic lesions could be detected at autopsy.

## DISCUSSION

In considering the differences in weight between the ferrets and mink used in these experiments for determining the m.l.d. of *botulinum* toxin, types A and B, it was seen that at least 2 animals in each group had a weight beyond the heaviest animal in the previous group. Hence, to evaluate the unit weight susceptibility of the mouse, mink, and ferret, one has to use the weight of the heaviest animal in that group which was killed by the highest dilution of the toxin (table 4).

When the m.l.d. per gram for the mouse (in each case) was determined by survival of the animal, it was apparent that ferrets and mink were more resistant on a weight basis than mice, both *per os* and intraperitoneally. It is interesting, however, that while both ferrets and mink were much more resistant to B toxin than A toxin by the intraperitoneal route, the contrary appeared to be the case when the administration was *per os*.

This fact appears significant when one considers that spores of type B *Cl. parabotulinum* are far more prevalent in the soil of the states of the middlewestern section of this country than are spores of the other types of this organism.[6, 10, 11, 12, 13, 14] The ferrets manifested considerably more individual variation in resistance to both types of the toxin than did the mink, notwithstanding the greater weight differences which were maintained among the ferrets

TABLE 3—Determination of Susceptibility of Female Mink to Botulinum Toxin Types A and B by the Intraperitoneal Route and Per Os

| A Toxin | | | | | B Toxin | | | | |
|---|---|---|---|---|---|---|---|---|---|
| No. animals | Mg. toxin | Vol. saline in inoc. | Route | Result | No. animals | Mg. toxin | Vol. saline in inoc. | Route | Result |
| 2 | 0.01 | 1.0 cc. | i.p. | Died eighteen hr. after treatment | 2 | 0.01 | 1.0 cc. | i.p. | No visible symptoms |
| 2 | 0.10 | 1.0 cc. | i.p. | Died eighteen hr. after treatment | 4 | 0.10 | 1.0 cc. | i.p. | Dead within three days |
| 2 | 0.50 | 1.0 cc. | i.p. | Died eighteen hr. after treatment | 2 | 0.50 | 1.0 cc. | i.p. | Dead after eighteen hr. |
| 2 | ... | 1.0 cc. | i.p. | No symptoms | 2 | 1.00 | 1.0 cc. | i.p. | Dead within three days |
| 2 | 20.00 | .... | *Per os* | No symptoms | 2 | 10.00 | 1.0 cc. | i.p. | Dead within three days |
| 2 | 30.00 | .... | *Per os* | No symptoms | 2 | ... | 1.0 cc. | i.p. | No symptoms |
| 2 | 40.00 | .... | *Per os* | Slight symptoms—recovered | 2 | 10.00 | 1.0 cc. | *Per os* | No symptoms |
| 2 | 50.00 | .... | *Per os* | Slight symptoms—recovered | 2 | 20.00 | 1.0 cc. | *Per os* | Dead fourth day |
| 2 | 60.00 | .... | *Per os* | One dead eighteen hr., the other recovered | 2 | 30.00 | 1.0 cc. | *Per os* | Dead fourth day |
| 1 | 70.00 | .... | *Per os* | Dead after four days | 2 | 40.00 | 1.0 cc. | *Per os* | One dead fourth day, 1 dead eighth day |
| 1 | 80.00 | .... | *Per os* | Prostrate after eight days—killed | 1 | 50.00 | 1.0 cc. | *Per os* | Symptoms — recovered |
| 2 | ... | .... | ..... | No symptoms | 1 | 60.00 | 1.0 cc. | *Per os* | Symptoms — recovered |
| | | | | | 1 | 70.00 | 1.0 cc. | *Per os* | Dead after five days |
| | | | | | 2 | .... | .... | .... | No symptoms |

The weight range in grams of the animals used, 600 to 1200. i.p. = intraperitoneal.

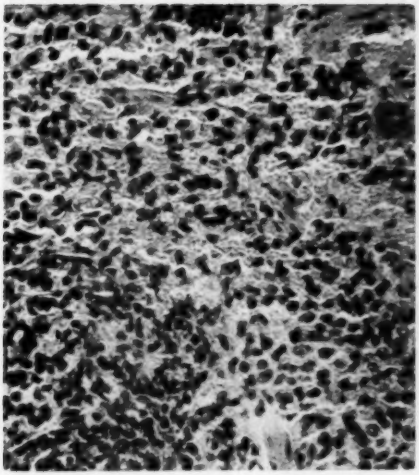

Fig. 5—Normal mink spleen. x 300. One giant cell is seen.

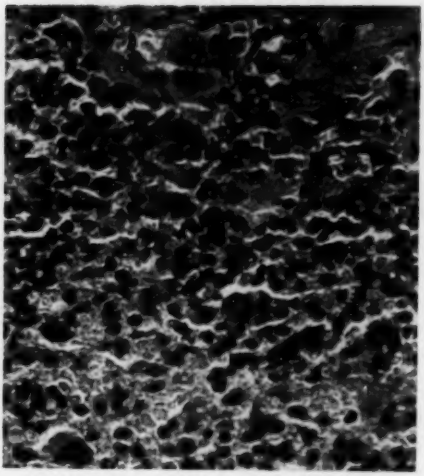

Fig. 6—Spleen of mink dead eight days after consumption of botulinum toxin type B. x 300. Giant cells generally seen in spleens of the poisoned animals.

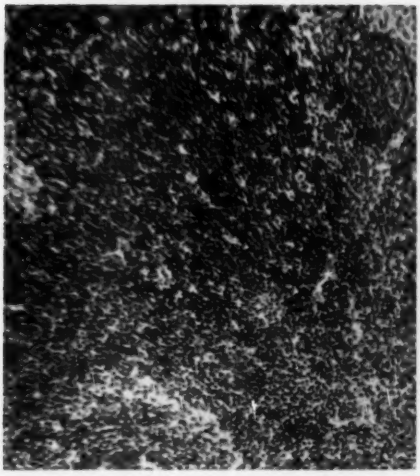

Fig. 7—Lymph node of normal mink. x 75. The lymph follicles well defined and numerous lymphocytic elements.

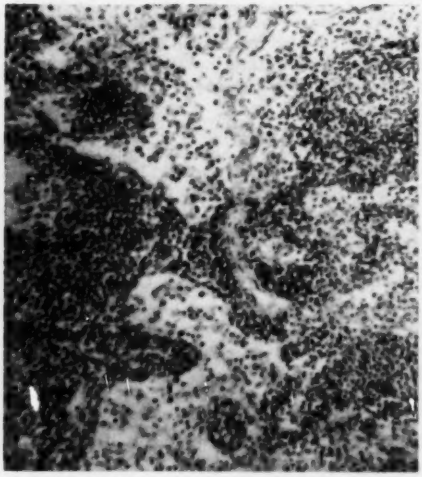

Fig. 8—Lymph node of mink dead eight days after consumption of botulinum toxin type B. x 75. Relatively few lymphocytic elements and indistinct follicular architecture.

than among the mink. The mink appeared to be definitely more resistant *per os* to type A toxin than to type B toxin. In the experiments where the toxin was given in the food, some of the variability may be ascribed to the way the animals took their food. All

TABLE 4—Comparison of the Susceptibility of Mouse, Ferret, and Mink to Botulinum Toxin Types A and B

| Route | Type toxin | Animal | Average weight, (Gm.) | m.l.d. | m.l.d./gram | Resistance per Gm. wt. based on mouse m.l.d. |
|---|---|---|---|---|---|---|
| i.p. | A | mouse | 20 | 0.000001 | 0.00000005 | 1 |
| i.p. | A | ferret | 1620 | 0.010000 | 0.00000060 | 12 |
| i.p. | A | mink | 840 | 0.010000 | 0.00000120 | 24 |
| i.p. | B | mouse | 20 | 0.000001 | 0.00000005 | 1 |
| i.p. | B | ferret | 1520 | 1.000000 | 0.00060000 | 1200 |
| i.p. | B | mink | 1000 | 1.000000 | 0.00010000 | 2000 |
| *Per os* | A | mouse | 20 | 0.010000 | 0.00050000 | 1 |
| *Per os* | A | ferret | | variable | variable | variable |
| *Per os* | A | mink | 1000 | 60-80 | 0.06-0.08000000 | 120-160 |
| *Per os* | B | mouse | 20 | 0.010000 | 0.00050000 | 1 |
| *Per os* | B | ferret | | variable | variable | variable |
| *Per os* | B | mink | 980 | 20.000000 | 0.02000000 | 40 |

i.p. = intraperitoneal.

food was not consumed at once, but small portions from time to time. The food may have been dragged or carried around, thus some was lost through the netting of the cage.

The two types of toxin caused similar symptoms in mink and ferrets. The first signs of poisoning were dullness, squinting of the eyes, urine soiling, and ataxia, with paralysis appearing as the intoxication proceeded. Urine soiling, however, seemed to be more marked in poisoning with the B than the A type of toxin. The mink generally manifested few symptoms, and most animals were found dead in prostrate position without any symptoms having been detected. This phenomenon may, to some extent, be ascribed to the very wild nature of these animals.

The time elapsing from injection to the development of symptoms and, also, the death rate seemed to be independent of the dose in the cases where the poisoning resulted in death. The time between inoculation and development of symptoms was generally shorter for the B type than for the A type of the toxin.

The gross lesions and the histological changes in the animals which died were the same for both types of toxin. The degree or severity of tissue alterations seemed to increase with the time of survival until death.

The spleen showed a most unusual picture typified by tissue exhaustion as the poisoning gained headway and in which the follicles and lymphatic tissue disappeared in conjunction with clumping of the reticulum and the appearance of many giant cells. A similar type of alteration was also found in the lymph nodes but it was not so marked in degree or extent.

The fairly constant finding of small hemorrhages in the cerebellum of dead animals may well account for the symptoms of ataxia so often seen. Significant alterations were not found in the intestinal tract. Inflammatory changes of the gastrointestinal tract were absent, although such changes in connection with *botulinum* poisoning have been reported in the literature.[2, 22] A group of animals was fed 40 mg. of toxin admixed with spoiled food to evaluate a factor which might contribute to such a change. It was assumed that this might simulate conditions of food decomposition that might be associated with *botulinum* intoxication on fur ranches. Evidence of inflammation was, however, not detected in the intestinal tract at autopsy of these animals.

To further orient these findings, 2 ferrets and 2 mink, fed fresh meat and apparently normal, were killed and the spleens and lymph nodes were examined. Both the normal spleen architecture and lymph node architecture, as described for the Carnivora, were found in each species (fig. 1, 3, 5, 7). The follicles seemed to be smaller and more numerous, with fewer lymphoblasts in the mink spleens than in the ferret spleens. The follicles, however, were very distinct in both species. A few giant cells were seen in the apparently normal spleens, but they ap-

peared to be the type with ring-forming nuclei.

The female ferrets seemed to be more susceptible than the males to the toxins. This difference may be ascribed in part to the smaller size of the females.

## CONCLUSIONS

Purified toxins of both types A and B of *Clostridium parabotulinum*[7, 8, 9] are quite stable at a temperature of 4 C., when kept in glass rubber-stoppered bottles in the dark.

Both the mink and ferret are susceptible to the A and B types of toxin, the mink is relatively much more resistant to type A than B toxin by way of the oral route, while the mouse and the ferret both are equally susceptible to the two types of toxin *per os*.

Fasting, extremes of environmental temperature, as well as reduced stomach acidity have not been found in these experiments to favor the action of the toxin.

Poisoning with *parabotulinum* toxin causes alteration of the spleen and, to some extent also, of the lymph nodes. This alteration is characterized by congestion of the spleen and necrosis in the center of the splenic follicles, follicular hyperplasia of mononuclear cells, disarrangement of reticulum, and the appearance of relatively numerous multinuclear giant cells as the poisoning progresses. Normal architecture is restored, however, shortly after clinical recovery from the poisoning.

Considering the fact that any single symptom, as well as any single necropsy finding, is by no means specific, the whole symptom complex, together with the pathological alterations and the circumstances under which the disease occurs, gives a fairly good basis for diagnosis of botulism in the mink and ferret.

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# Studies on the Hematology of the Thoroughbred Horse. V. Sucklings

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PRESENT INFORMATION on the hematology of Thoroughbred foals rests mainly on the study made by MacLeod, Ponder, Aitken, and Brown,[1] who reported erythrocyte and leukocyte counts, hemoglobin content, and differential white cell percentages for a total of 12 sucklings. Reference values for Thoroughbred foals are of primary importance to this laboratory because of investigations conducted on the effects of anthelmintic treatment on the physical well-being of horses.[2, 3, 4] In addition, such information is important in the diagnosis of cases of so-called jaundiced or icteric foals and in their prognosis, as well as in the diagnosis of other anemic conditions.

A series of studies on the hematology of Thoroughbred mares in foal, weanlings, stallions, and barren mares has been presented previously by this laboratory.[5, 6, 7, 8] These studies each reported values for the erythrocyte and leukocyte counts, hemoglobin content, packed volume of erythrocytes, mean corpuscular volume, mean corpuscular hemoglobin concentration, and differential counts, together with the ranges, median, mean and standard error, and standard deviation for each value. This series of studies, based on working groups of horses as they were encountered in veterinary practice at breeding farms, necessarily remained incomplete. Data now have been accumulated on the blood picture of Thoroughbred sucklings, another working group of horses at a nursery. The entire picture of the hematology of Thoroughbreds would require, of course, a study of yearlings and horses in training.

The population of horses used in the present study is more restricted, and at the same time more intensive, than in the previous studies. Twenty-eight foals, out of 19 mares, were examined over a three-year period. A series of blood samples were obtained from each suckling in a given year, so that the data presented rest on 217 individual examinations.

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The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

## METHODS

The details of the methods used in this study are given in the first paper of the series.[5]

This study was conducted with three successive foal crops at one nursery; foals of 1948, 1949, and 1950 are included. All samples were collected between 8 and 9 a.m., before the foals were turned out. None of the animals was twitched and every effort was made to take the blood sample without exciting the foals. The youngest foal bled was two days old; the oldest suckling was 182 days old. Because the samples were taken in series, the data have been presented by chronological age of the foals studied.

## RESULTS AND DISCUSSION

The data relating to the range, median, mean and standard error, and standard deviation for erythrocytes and leukocytes, packed cell volume, mean cell volume, mean corpuscular hemoglobin concentration, and hemoglobin are given in table 1. The same measurements for total leukocyte counts are given in table 2, along with measurements of the differential leukocyte percentages.

Because the above measurements of individual values are not given by MacLeod and his co-workers,[1] comparisons between his data and ours can serve little useful purpose. Their data on erythrocytes per cubic millimeter all fall within one standard deviation of ours; this is also true of their differential values for neutrophils and lymphocytes. Our eosinophil percentages agree with theirs through our 9- to 12-week-old category but not thereafter. Our records of hemoglobin in grams per 100 ml. are consistently lower than the 14 Gm. given by MacLeod *et al.* The percentage of monocytes (7.0) recorded by the latter study is greatly in excess of the percentages recorded by us.

The immediate decrease in total erythrocyte count recorded in newborn human beings was not obtained in this study of Thoroughbred sucklings. The youngest foal bled was two days old.

The age groupings of the foals shown in tables 1 and 2 are purely arbitrary. The groupings of 1 to 4-, 5 to 8-, 9 to 12-, 13 to 19-, and 20 to 26-week-old foals were made in order to demonstrate a number of interesting occurrences. First, the average number of erythrocytes per cubic millimeter appears to increase from the youngest group at 11,120,000 per cubic millimeter to a high of 12,640,000 in foals 9 to 12 weeks old. Thereafter, the average declines. An increase and decrease of hemoglobin in grams per 100 ml. and the percentages of packed cell volumes accompanied the erythrocyte counts. The mean cell volume was found to decline in the first twelve weeks after birth and then increased slightly. No apparent change occurred in the mean corpuscular hemoglobin concentration.

The number of total leukocytes in circulating blood were found to show a continuous, gradual increase. Foals 1 to 4 weeks old averaged 8,250 per 1 cmm; foals 5 to 9 weeks old averaged 10,850 per 1 cmm., while those at 9 to 12 weeks averaged 13,750. Foals 20 to 26 weeks old had an average of 14,050 (table 2). These figures exceed averages previously reported by this laboratory *(ibid.)* for stallions, mares in foal, barren mares, and weanlings. The situation, including a moderate decline in the number of neutrophils, is similar to data recorded by Kracke for newborn infants.[9]

TABLE 1—Blood Picture of Healthy Thoroughbred Foals in Central Kentucky

| Age grouping and No. samples | Measure | Erythrocytes ($10^6$/cmm.) | Hemoglobin (Gm./100 ml.) | Packed volume of erythrocytes (%) | Leukocytes ($10^3$/cmm.) | Mean corpuscular volume (cμ) | Mean corpuscular hemoglobin concentration (%) |
|---|---|---|---|---|---|---|---|
| 1 to 4 weeks (10 foals, 15 samples) | Range | 9.09-14.20 | 11.0-13.9 | 33.0-43.5 | 4.80-11.35 | 27.46-37.80 | 31.94-34.84 |
| | Median | 11.00 | 12.0 | 36.0 | 8.25 | 32.72 | 33.23 |
| | Arithmetic mean and s.e.* | 11.12±0.384 | 12.07±0.255 | 36.19±0.794 | 8.36±0.519 | 33.00±0.769 | 33.35±0.238 |
| | Standard deviation | 1.48±0.282 | 0.98±0.180 | 3.07±0.561 | 2.01±0.364 | 2.98±0.544 | 0.92±0.168 |
| 5 to 8 weeks (14 foals, 25 samples) | Range | 9.25-15.21 | 10.9-14.1 | 32.7-44.0 | 6.30-15.70 | 27.27-35.70 | 30.63-36.08 |
| | Median | 12.30 | 12.7 | 37.9 | 10.85 | 30.31 | 33.33 |
| | Arithmetic mean and s.e. | 12.19±0.304 | 12.6±0.204 | 38.04±0.699 | 10.61±0.583 | 31.48±0.471 | 33.21±0.252 |
| | Standard deviation | 1.52±0.215 | 1.02±0.145 | 3.49±0.495 | 2.86±0.412 | 2.36±0.333 | 1.27±0.179 |
| 9 to 12 weeks (19 foals, 30 samples) | Range | 9.60-15.00 | 10.7-16.0 | 32.1-49.0 | 9.40-21.60 | 26.25-33.99 | 29.88-36.34 |
| | Median | 12.64 | 13.0 | 39.0 | 13.75 | 30.0 | 33.16 |
| | Arithmetic mean and s.e. | 12.81±0.292 | 12.9±0.214 | 38.8±0.638 | 13.64±0.432 | 30.42±0.389 | 33.35±0.266 |
| | Standard deviation | 1.59±0.206 | 1.17±0.151 | 3.49±0.451 | 2.37±0.305 | 2.13±0.276 | 1.45±0.187 |
| 13 to 19 weeks (18 foals, 91 samples) | Range | 9.30-15.20 | 10.1-15.9 | 31.0-47.0 | 9.35-19.25 | 27.50-34.92 | 29.44-42.50 |
| | Median | 11.59 | 12.0 | 36.0 | 13.45 | 30.89 | 33.42 |
| | Arithmetic mean and s.e. | 11.83±0.141 | 12.2±0.125 | 36.3±0.351 | 13.67±0.225 | 30.80±0.161 | 33.60±0.234 |
| | Standard deviation | 1.35±0.099 | 1.19±0.088 | 3.35±0.248 | 2.14±0.159 | 1.53±0.114 | 2.01±0.167 |
| 20 to 26 weeks (11 foals, 56 samples) | Range | 8.52-14.19 | 9.9-13.8 | 28.5-41.7 | 9.95-20.50 | 24.67-37.15 | 29.55-36.66 |
| | Median | 10.50 | 11.7 | 34.5 | 14.05 | 31.7 | 33.23 |
| | Arithmetic mean and s.e. | 11.11±0.193 | 11.7±0.119 | 35.1±0.396 | 14.53±0.304 | 31.68±0.322 | 33.38±0.197 |
| | Standard deviation | 1.44±0.136 | 0.89±0.085 | 2.97±0.280 | 2.49±0.215 | 2.41±0.228 | 1.47±0.139 |
| Total | Range | 8.52-15.21 | 9.9-16.0 | 23.5-49.0 | 4.8-21.6 | 24.67-37.8 | 29.44-42.50 |
| | Arithmetic mean and s.e. | 11.77±0.096 | 12.2±0.073 | 36.54±0.220 | 13.12±0.153 | 31.2±0.14 | 33.45±0.113 |
| | Standard deviation | 1.42±0.068 | 1.07±0.052 | 3.24±0.156 | 2.43±0.108 | 2.06±0.099 | 1.66±0.079 |

*Standard error.

## SUMMARY

1) The following over-all mean blood values with their standard errors are recorded from a series of 217 individual examinations of 28 Thoroughbred sucklings: erythrocytes per cubic millimeter, 11.77±0.096 million; hemoglobin, grams per 100 ml., 12.2±0.073; packed volume of erythrocytes, 36.54±0.22 per cent; mean corpuscular volume, 31.2±0.14 cμ; mean corpuscular hemoglobin concentration, 33.45±0.113 per cent; and leukocytes per cubic millimeter, 13.12±0.153 thousand.

2) The differential leukocyte counts have the following mean percentages and standard errors: neutrophils, 53.26±0.742; lymphocytes, 42.16±0.742; monocytes, 2.37±0.119; eosinophils, 2.1±0.118; and basophils, 0.07±0.027.


**TABLE 2—Total Leukocyte Counts and Differential Leukocyte Percentages in Blood of Healthy Thoroughbred Foals in Central Kentucky**

| Age grouping and No. samples | Measure | Leukocytes* ($10^3$/cmm.) | Neutrophils (%) | Lymphocytes (%) | Monocytes (%) | Eosinophils (%) | Basophils (%) |
|---|---|---|---|---|---|---|---|
| 1 to 4 weeks (10 foals, 12 samples) | Range | 4.80-11.35 | 37.5-80.5 | 19.0-62.0 | 0-3.5 | 0-1.0 | ..... |
| | Median | 8.25 | 59.5 | 37.5 | 1.0 | 0.0 | ..... |
| | Arithmetic mean and s.e.** | 8.36±0.519 | 59.17±3.674 | 39.08±3.684 | 1.46±0.309 | 0.29±0.132 | ..... |
| | Standard deviation | 2.01±0.364 | 12.41±2.598 | 12.72±2.605 | 1.07±0.218 | 1.07±0.218 | ..... |
| 5 to 8 weeks (9 foals, 13 samples) | Range | 6.30-15.70 | 41.5-73.5 | 23.5-55.0 | 0-4.0 | 0-1.5 | 0-0.5 |
| | Median | 10.85 | 59.5 | 37.5 | 2.0 | 0.0 | 0.0 |
| | Arithmetic mean and s.e. | 10.61±0.583 | 59.11±2.499 | 38.62±2.916 | 1.85±0.313 | 0.38±0.145 | 0.04±0.039 |
| | Standard deviation | 2.86±0.412 | 9.90±2.030 | 10.49±2.058 | 1.13±0.221 | 0.51±0.099 | 0.14±0.027 |
| 9 to 12 weeks (14 foals, 24 samples) | Range | 9.40-21.60 | 33.0-62.5 | 34.5-64.0 | 0-4.0 | 0-9.5 | 0-0.5 |
| | Median | 13.75 | 48.0 | 47.0 | 2.0 | 0.5 | 0.0 |
| | Arithmetic mean and s.e. | 13.64±0.432 | 48.56±1.785 | 48.27±1.429 | 1.9±0.188 | 1.12±0.400 | 0.1±0.014 |
| | Standard deviation | 2.37±0.305 | 8.75±1.262 | 8.37±1.237 | 0.92±0.133 | 1.96±0.283 | 0.07±0.010 |
| 13 to 19 weeks (17 foals, 82 samples) | Range | 9.35-19.25 | 26.0-70.5 | 24.0-73.5 | 0-7.5 | 0-7.0 | 0-0.5 |
| | Median | 13.45 | 55.5 | 41.0 | 2.5 | 2.5 | 0.0 |
| | Arithmetic mean and s.e. | 13.67±0.225 | 53.2±1.161 | 42.0±1.175 | 2.38±0.153 | 2.35±0.154 | 0.05±0.056 |
| | Standard deviation | 2.14±0.159 | 10.51±0.821 | 10.64±0.831 | 1.42±0.108 | 1.39±0.109 | 0.51±0.039 |
| 20 to 26 weeks (9 foals, 53 samples) | Range | 9.95-20.50 | 30.0-82.0 | 15.0-68.5 | 0-13.5 | 0-10 | 0-1.0 |
| | Median | 14.05 | 51.5 | 41.5 | 2.0 | 2.5 | 0.0 |
| | Arithmetic mean and s.e. | 14.53±0.304 | 52.7±1.324 | 41.2±1.296 | 2.9±0.318 | 3.0±0.282 | 0.12±0.035 |
| | Standard deviation | 2.49±0.215 | 9.65±0.936 | 9.38±0.917 | 2.29±0.225 | 2.05±0.199 | 0.25±0.025 |
| Total | Range | 4.8-21.6 | 26.0-82.0 | 15.0-73.5 | 0-13.5 | 0-10.0 | 0-1.0 |
| | Arithmetic mean and s.e. | 13.12±0.153 | 53.26±0.742 | 42.16±0.742 | 2.37±0.119 | 2.10±0.118 | 0.07±0.027 |
| | Standard deviation | 2.43±0.108 | 10.06±0.524 | 10.06±0.524 | 1.61±0.084 | 1.60±0.083 | 0.37±0.019 |

*Based on counts of table 1. **Standard error.

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# Susceptibility to Newcastle Disease Infection of Chickens Vaccinated with a Killed Virus Vaccine

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OBSERVATIONS ON the use of commercially available, killed virus Newcastle disease (ND) vaccines by Adler *et al.*,[1] Schoening *et al.*,[2] and Bolin and Eveleth[3] indicated that exposure to virulent Newcastle disease virus (NDV) caused both paralytic symptoms and fatal infection of some previously vaccinated chickens. From their reports, it was evident that killed virus ND vaccines did not protect uniformly against NDV infection. The authors are not aware of experiments reporting a critical clinical observation and serological study of all members of a killed virus-vaccinated chicken population following experimental exposure to NDV. The study reported here was undertaken to ascertain if vaccination of chickens with a killed virus ND vaccine would induce a state of refractivity adequate for preventing infection with NDV.

## MATERIALS AND METHODS

The chickens used in the first series of experiments (table 1) were hatched from eggs laid by hens which were vaccinated intranasally ten months previously with the B1 strain[4] of NDV. There was neither clinical nor serological evidence of naturally occurring ND infection in the flock prior to vaccination or during the interim between the vaccination and collection of eggs for hatching. These chickens, therefore, probably received little or no passive protection from the egg yolk.[4, 7] The chickens employed in the second experiment (table 2) were hatched from eggs laid by hens which had no known infection or vaccination with NDV. The chickens were started in electrically heated brooders and, when 3 weeks old, were transferred to growing batteries. A commercial baby chick starting mash was fed throughout the experiment.

The vaccine employed in these experiments, a formalin-inactivated, aluminum hydroxide-adsorbed product of chicken embryo origin, was purchased on the open market. It was used approximately six months prior to the label-expiration date. The vaccine was injected into the pectoral muscles, using doses of 0.5 and 1.0 cc. The vaccinated and control chickens were raised together in the same battery until the challenge exposures were made.

The GB strain of NDV employed for challenge exposure consisted of infective amnio-allantoic fluid harvested from dead embryos of the first and second passages of a reisolation from a chicken that died from contact exposure. Since facilities were not available for titration of the challenge virus in chickens, only chicken embryos were used for determining the infectivity of the inoculum. Also, it obviously was not possible to control the quantity of virus acquired by chickens exposed by direct contact and airborne contact exposure, which should simulate natural infection among battery-grown chickens. Unvaccinated control chickens were employed to obtain information on the pathogenicity of the dose of virus used for the intranasal challenge exposures and for mortality percentages resulting from contact exposure. For the direct contact exposures, chickens were placed in the same section of a growing battery with an equal number of intranasally inoculated birds. The total number was 6 or 8 chickens for each section of the battery. For the airborne contact exposures, a similar number of chickens was placed in separate sections of a battery or in a separate battery. After exposure, the chickens were observed daily, and an appraisal was made of the general condition and presence or absence of respiratory symptoms. Procedures for the hemagglutination-inhibition (HI) test and the serum-neutralization (SN) tests were described previously.[5]

## RESULTS

A group of experiments employing single and double doses of the killed virus ND vaccine are summarized in table 1. In all instances, the dose of vaccine was 1.0 cc. injected into the pectoral muscles. One lot of

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chickens was vaccinated at 4 weeks of age. Four weeks later, they were divided into groups, and 30 chickens were inoculated intranasally with $10^6$ embryo lethal $dose_{50}$ ($l.d._{50}$) of the GB strain of NDV. A group of 15 chickens was exposed by placing them in direct contact with the intranasally inoculated chickens, and another group of 15 chickens was exposed in separate sections of the batteries so that infection would be initiated by airborne transmission. Another lot of 4-week-old chickens was vaccinated, and a similar number of chickens, 30, 15, and 15, respectively, were subjected to intranasal inoculation, direct contact, and airborne contact exposure three weeks later. Another group of chickens received two doses of vaccine, one at 4-weeks of age and one at 5-weeks of age. The challenge exposure was made two weeks later, three and two weeks following the first and second doses of vaccine, respectively. Mortality and the development of nervous symptoms occurred in previously vaccinated chickens in each of the groups listed in table 1. In some instances, the percentage of combined mortality and nervous symptoms resulting from contact and airborne exposure exceeded that from intranasal inoculation. Similarly, the percentage of mortality resulting from airborne contact exposure exceeded that from intranasal inoculation in the control chickens. Daily examination of the chickens during a three-week period following exposure disclosed symptoms of respiratory infection (sneezing, coughing, and râles) in almost every chicken of the vaccinated group. In the majority of the surviving chickens, the infection appeared to be limited to the respiratory tract, but symptoms of systemic infection occurred in some.

Serum-neutralization and HI tests were made on serums obtained one day before challenge exposure of the two lots of chickens receiving single doses of vaccine. The SN indexes varied from zero to 100 embryo $l.d._{50}$, the majority of chickens having zero values. Hemagglutination-inhibition titers were less than 1:5 by the *beta* procedure in all of the vaccinated chickens. Three weeks after exposure, the serum of every surviving vaccinated chicken completely inhibited agglutination at 1:640, the highest serum dilution tested. Prechallenge serums from the chickens receiving two doses of vaccine had HI titers of less than 1:5 for all except 1 chicken, which had a titer of 1:20. Three weeks after challenge exposure, all survivors had HI titers of 1:640 or greater.

To eliminate the possibility of interference with immunization by passively transferred yolk antibody which may have been obtained by chickens in the experiments cited above, another vaccination trial was made using chickens hatched from nonvaccinated susceptible hens. In this study, the chickens received four consecutive intramuscular injections of killed virus ND vaccine. The doses were 0.5, 0.5, 1.0, and 1.0 cc., respectively, at the ages of 1, 2, 3, and 4 weeks. Two weeks following the last dose of vaccine, the chickens were exposed to virulent NDV. Twenty chickens were inocu-

TABLE 1—Results Obtained from Three Methods of Challenge Exposure of Chickens† Previously Vaccinated with a Killed Virus Newcastle Disease Vaccine

| No. chickens | Age at vaccination (weeks) | Weeks from vaccination to challenge | Age of chickens when challenged (weeks) | Method of exposure | No. deaths | No. with nervous symptoms** | Survivors with serological evidence of infection |
|---|---|---|---|---|---|---|---|
| 30 | 4 | 4 | 8 | IN | 3 | 1 | 26 |
| 15 | 4 | 4 | 8 | DC | 1 | 4 | 10 |
| 15 | 4 | 4 | 8 | AC | 4 | 0 | 11 |
| 30 | 4 | 3 | 7 | IN | 3 | 1 | 26 |
| 15 | 4 | 3 | 7 | DC | 4 | 1 | 10 |
| 15 | 4 | 3 | 7 | AC | 2 | 0 | 13 |
| 20 | 4 and 5* | 3 and 2 | 7 | IN | 7 | 2 | 11 |
| 20 | 4 and5 | 3 and 2 | 7 | AC | 3 | 3 | 14 |
| 32 | Unvaccinated controls | ..... | 7 | IN | 28 | 1 | 3 |
| 20 | Unvaccinated controls | ..... | 7 | AC | 19 | 0 | 1 |

†Chickens used in these experiments were hatched from hens vaccinated intranasally with the B1 strain of Newcastle disease virus ten months previously. *Two consecutive doses of vaccine separated by a seven-day interval. **Chickens showing symptoms of impaired function of the nervous system at three weeks after exposure. IN = intranasal inoculation. DC = direct contact exposure. AC = airborne contact exposure.

lated intranasally with $10^8$ embryo $l.d._{50}$ of the GB strain of NDV, 20 chickens were exposed by direct contact with those inoculated intranasally, and 20 chickens were subjected to airborne contact exposure. Examination of table 2 shows that there was only a slight difference in the total mortality and nervous symptoms among the vaccinated chickens from the three methods of exposure. Also, the total mortality and nervous symptoms from direct contact exposure exceeded that from intranasal inoculation. Individual examination of the surviving chickens disclosed that every chicken developed symptoms of respiratory infection from five to twelve days after exposure, regardless of the method of exposure to which each was subjected. Determination of the HI activity of individual samples of serum from the vaccinated chickens obtained on the day prior to exposure resulted in HI titers ranging from 1 : 80 to 1 : 320. The average preëxposure HI titer for the group inoculated intranasally was 1 : 186, that for the group exposed by direct contact was 1: 200, and that for the group exposed by airborne infection was 1: 260. Three weeks after exposure, the HI titers varied from 1: 1,280 to 1: 10,240. The average postchallenge HI titers were 1: 3,256, 1: 2,470, and 1: 3,008, respectively, for the intranasal, direct contact, and airborne contact groups.

## DISCUSSION

In the experiments reported here, neither single nor multiple vaccination of chickens less than 5 weeks old with a killed virus ND vaccine entirely prevented fatal infection or symptomatic infection of the nervous system by the GB strain of NDV. The percentage of mortality plus nervous symptoms resulting from challenge exposure was comparable in groups of chickens previously receiving a single dose of vaccine and in chickens receiving vaccine at weekly intervals until two or four doses were given. Also, no appreciable difference in mortality was obtained following vaccination and challenge of young chickens hatched from nonvaccinated susceptible hens and from hens vaccinated intranasally ten months previously with the B1 strain of NDV.

In a previous study,[6] it was observed that chickens vaccinated intranasally with the B1 strain of NDV rapidly lost their HI titers and became susceptible to infection of the respiratory tract within seven weeks after vaccination. The B1-vaccinated chickens resisted fatal infection, but developed symptoms of respiratory infection, yielded positive isolations of NDV from tracheal swabs, and showed a marked increase of HI titers following intranasal inoculation and contact exposure. In the light of these findings, we considered it unnecessary to attempt virus isolation from the chickens employed in this study. A marked increase of the HI titer and the occurrence of symptoms of respiratory infection were regarded as adequate evidence that the challenge virus had become established and multiplied in the respiratory tract of the chickens previously vaccinated with the killed virus ND vaccine. Although the chickens employed in the first series of experiments (table 1) were not examined individually, critical observation indicated that almost

**TABLE 2—Results Obtained from Three Methods of Challenge Exposure of Chickens† Previously Vaccinated with Four Doses of a Killed Virus Newcastle Disease Vaccine**

| No. chickens | Age at vaccination (weeks) | Weeks from last vaccination to challenge | Age of chickens when challenged (weeks) | Method of exposure | No. deaths | No. with nervous symptoms** | Survivors showing symptoms and serological evidence of infection |
|---|---|---|---|---|---|---|---|
| 20 | 1, 2, 3, 4* | 2 | 6 | IN | 4 | 1 | 15 |
| 20 | 1, 2, 3, 4 | 2 | 6 | DC | 6 | 0 | 14 |
| 20 | 1, 2, 3, 4 | 2 | 6 | AC | 3 | 1 | 16 |
| 20 | Unvaccinated controls | .... | 6 | IN | 18 | 1 | 1 |
| 20 | Unvaccinated controls | .... | 6 | AC | 19 | 1 | 0 |

†The chickens used in this experiment were hatched from susceptible hens. *The vaccine doses were 0.5, 0.5, 1.0, and 1.0 cc., respectively, at ages of 1, 2, 3, and 4 weeks. IN = intranasal inoculation. DC = direct contact exposure. AC = airborne contact exposure. ** = chickens showing symptoms of impaired function of the nervous system at three weeks after exposure.

every bird developed symptoms of respiratory infection. Without exception, every surviving chicken previously receiving one or two doses of killed virus ND vaccine had a marked increase in HI titer, providing evidence of invasion of the tissues by the challenge virus. With both the single and double doses of killed virus ND vaccine, protection against fatal infection was obtained in the absence of demonstrable circulating antibody.

All chickens of the group receiving four doses of vaccine (table 2) developed significant HI antibody. Live virus challenge employing the GB strain by intranasal inoculation and contact exposure, however, resulted in fatal infection of chickens having HI titers ranging from 1:80 to 1:320. Daily examination of the individual chickens following challenge exposure revealed symptoms of respiratory infection in every surviving chicken. Also, all survivors showed a great increase of HI titer at three weeks after exposure, providing further evidence of an active infection. Apparently, such infection with the GB strain of NDV may occur at all periods after vaccination with killed virus ND vaccines possessing immunizing qualities comparable to that of the vaccine used in these experiments.

The failure of the killed virus ND vaccine to provide protection against infection of the respiratory tract in these experiments suggests limitations of products of this type in the control of ND. The absence of protection against respiratory infection following administration of four doses of vaccine given at weekly intervals indicates that it is unlikely that field vaccination with a single dose of killed virus ND vaccine could be expected to establish immunity adequate for preventing infection of the respiratory tract by virus strains having the infective potential of the GB strain. The possibility that high pathogenicity may not be necessary for such infection to occur was shown by successful reinfection by NDV strains B1 and Ky-50 in chickens previously vaccinated intranasally with the B1 strain.[6] Although protection against fatal infection may be obtained as a result of killed virus vaccination, the procedure may not provide an effective barrier against acquisition of active infection by previously vaccinated birds and possibly further transmission to similarly or fully susceptible chickens. Killed virus ND vaccines having antigenicity comparable to that used in these experiments apparently will not provide a fully effective means of limiting or preventing the spread of outbreaks of ND, but they may be useful in reducing losses. It appears that a similar, though probably lesser, degree of susceptibility to respiratory infection exists in chickens four to eight weeks after intranasal vaccination with the living B1 strain of NDV.[6] Conditions under which similar infection may occur following vaccination with the more virulent, wing-web, live virus vaccines apparently have not been determined.

## SUMMARY

Chickens were found to be susceptible to infection of the respiratory tract by the GB strain of Newcastle disease virus at periods of three to four weeks after single doses of a killed virus Newcastle disease vaccine intramuscularly and at two weeks after four consecutive doses at weekly intervals. Although a high percentage of vaccinated birds were refractory to fatal infection, every chicken exposed by intranasal inoculation, direct contact exposure, or airborne contact exposure acquired infection of the respiratory tract.

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